Isolation, characterization and identification of *Actinobacteria* of Mangrove ecosystem, Bhitarkanika, Odisha

PROJECT SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIEMENT OF MASTER OF SCIENCE IN LIFE SCIENCE

By

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CERTIFICATE

This is to certify that the project report titled **"Isolation, characterization and identification of *Actinobacteria* of Mangroove ecosystem, Bhitarkanika, Odisha."** submitted by Ms Priyanka Kishore to the Department of Life Science, National Institute of Technology, Rourkela in partial fulfilment of the requirement for the degree of Master of Science in Life Science is a bonafide record of work carried out by her under my supervision. The contents of this report in full or parts have not been submitted to any other Institute or University for the award of any degree or diploma.

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This project is by far the most significant accomplishment in my life and it would not have been impossible without people who supported me and believed in my caliber.

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Last, but not the least, I would thank the Almighty and my parents, whose dedicated and untiring efforts towards me has brought me at this stage of my life.

Priyanka Kishore
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DECLARATION

I, Miss Priyanka Kishore, M. Sc. Life Science, 4th semester, Department of Life Science, NIT, Rourkela hereby declare that my project work titled "**Isolation, characterization and identification of *Actinobacteria* of Mangrove ecosystem Bhitarkanika, Odisha"** is original and no part of this work has been submitted for any other degree or diploma. All the given information is true to best of my knowledge.

(Priyanka Kishore)

Date:

Place:

ABBREVIATION

%	Percentage
CFU	Colony forming unit
Min	minute
Mg	Milligram
µg	Microgram
gm	Gram
^{0}C	Degree Celsius
^{0}F	Degree Ferhenite
Km	Kilometer
m	Meter
µm	Micrometer
mm	Millimeter
ml	Milliliter
cm	Centimeter

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ABSTRACT

In the present study 15 strains were isolated from the Mangrove ecosystem of Bhitarkanika , Odisha. All the strains have shown white and gray aerial mass colour . Melanoid formation is shown by seven strains and only four strains namely BS, C8, C9 and MB3 shows the distinctive (1) character of reverse side pigments. Spiral spore chain morphology has been shown by 10 strains and only one strain has shown biverticillus spira. Strain BS has shown Rectus form. For the spore surface morphology of three strains SEM analysis is done, among them one strain CS has shown spiny and another two Strain B2 and C11 has shown smooth surface. Assimilation of rhamnose, Inositol and mannitol was better than other carbon sources. Strain B2, C8, C9, MB1, MB2 and MB5 has shown good enzymatic activity. Strain D1 has shown good antimicrobial activity against pathogenic strains of *Pseudomonas, E.coli, Klebsiella, Bacillus,* and *Proteus.* Results were interpreted for the identification of strain species from the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project).

INTRODUCTION

Mangrove forests are among the world's most productive ecosystem that enriches coastal waters, yields commercial forest products, protect coastlines and support coastal fisheries. However, mangroves exist under condition of high salinity, extreme tides, strong winds, high temperature and muddy, anaerobic soils. There may be no other group of plants with such highly developed morphological, biological, ecological and physiological adaptations to extreme conditions. Mangroves are woody plants that grow at the interface between land and sea in tropical and subtropical latitudes. These plants, and the associated microbes, fungi, plants and animals, constitute the mangrove forest community or mangal. (Kathiresan and Bingham, 2001). Mangroves provide nursery habitat for commercial fish, crustaceans and wildlife species that contribute to sustaining the survival of local fish and shellfish populations (Brown, 1997).

Mangrove systems support a very wide range of wildlife species including crocodile, birds (Figure 1), tigers, deer, monkeys and honey bees (Field, 1998). Many animals find shelter either in the roots and branches of mangroves. Mangroves serve as rookeries, or nesting areas, for coastal birds such as brown pelicans and roseate spoonbills. Many migratory species depend on mangroves for part of their seasonal migrations. Mangroves maintain coastal water quality by abiotic and biotic retention, removal and cycling of nutrients, pollutants, and particulate matter from land based sources, filtering these materials from water before they reach seaward coral reef and seagrass habitats (Lal, 1990). Mangrove root systems slow water flow, facilitating the deposition of sediment. Their adaptation to salinity condition becomes possible due to their resistance to concentration of salt, entering roots and secretion of salts from their leaves. Many mangroves have stilt root, which are aerial and acts as anchoring structure to withstand wave action (Mohanty, 1992). Some mangroves have inverted wedge like projections on the ground from the underground root system, called pneumatophores. The plants breathe in oxygen through the pores of pneumatophores during prolonged time of submergence of the root system (Fig. 1). The mangrove eco-system is highly fragile and the essential factors for its

maintenance are fresh water influx from adjoining land and tidal inflow from the sea (The State of Forest Report, 1999 and 2001).

Marine environments are largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites (Baskaran *et al*., 2011). Among such Microorganisms, actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities (Bredholt *et al.*, 2008). The demand for new antibiotics continues to grow due to the rapid emerging of multiple antibiotic resistant pathogens causing life threatening infection. Now a day's considerable progress is being continuing within the fields of chemical synthesis and in the field of engineered biosynthesis of antibacterial compounds. So, the nature still remains the richest and the most versatile source for new antibiotics. (Kpehn and Carter, 2005; Baltz, 2006; Pelaez, 2006).

Traditionally, actinomycetes have been isolated from the terrestrial sources only and the first report of mycelium forming actinomycetes being recovered from marine sediments appeared several decades ago (Weyland, 1969). Recently, the marine derived actinomycetes are recognized as a source of novel antibiotic and anticancer agent with unusual structure and properties (Jensen *et al.*, 2005). Actinomycetes represent a ubiquitous group of microbes widely distributed in natural ecosystems around the world and very significance on the recycling of Organic matter (Srinivasan *et al.*, 1991).

Figure 1. Typical mangrove ecosystem.

Actinomycetes are gram positive bacteria, with a high guanine (G) plus cytosine (C) ratio in their DNA (>55mol %), which are phylogenetically related from the evidence of 16S ribosomal cataloguing and DNA: rRNA pairing studies (Goodfellow and Williams, 1983). The name “Actinomycetes” was derived from Greek “atkis” (a ray) and “mykes” (fungus), and has features of both Bacteria and fungi (Das *et al*., 2008). Actinomycetes are soil organisms which have characteristics common to bacteria and fungi and yet possess sufficient distinctive features to delimit them into a distinct category. In the strict taxonomic sense, actinomycetes are clubbed with bacteria in the same class of Schizomycetes but confined to the order Actinomycetales (Kumar *et al*., 2005). The actinomycetes are a group of bacteria which possess many important and interesting features. They are of considerable value as producers of antibiotics and of other therapeutically useful compounds. They exhibit a range of life cycles which are unique among the prokaryotes and appear to play a major role in the cycling of organic matter in the soil ecosystem (Veiga *et al*., 1983). Therefore, actinomycetes hold a prominent position due to their diversity and proven ability to produce new compounds, because the discovery of novel antibiotic and non-antibiotic lead molecules through microbial secondary metabolite screening is becoming increasingly important.

Actinomycetes population considered as one of the major group of soil population (Kuster, 1968), but after that it is being isolated from a diverse range of marine samples, including sediments obtained from deep-sea (Walker *et al*., 1975; Colquhoun *et al*., 1998), even from greatest depth- Mariana Trench (Takami *et al*., 1997; Pathom-aree *et al*., 2006), and also in the vicinity of hydrothermal vents (Murphy and Hill, 1998). It is now accepted that actinomycetes can be indigenous to the marine environment and that this environment is likely to yield many unusual actinomycetes that have great potential as producers of novel antibiotics and other compounds. These marine actinomycetes play important ecological roles, similar to their saprophytic relatives in soils, perhaps substantially impacting the cycling of complex carbon substrates in benthic ocean habitats (Mincer *et al*., 2002). However, a well-defined biodiversity and taxonomic study of actinomycetes is important to understand actinomycetes from the marine environment (Das *et al*., 2008). On agar

plates actinomycetes can be easily distinguished from true bacteria. Unlike slimy distinct colonies of true bacteria which grow quickly, they appear slowly and show powdery consistency and also stick firmly to agar surface.

Actinomycetes have gained prominene in recent years because of their potential for producing antibiotics (Kumar *et al*., 2005). Streptomycin, gentamicin, rifamycin are some of the antibiotics which are in use presently and erythromycin are the product of actinomycetes. The actinomycetes are important in the field of pharmaceutical industries and also the agriculture. Previous study showed that actinomycetes isolated from Malaysia soil have the potential to inhibit the growth of several plant pathogens (Jeffrey *et al*., 2007). Oskay *et al*. (2004) also reported about the ability of actinomycetes isolated from Turkey's farming soil they have the ability to inhibit *Erwinia amylovora* a bacteria that cause fireblight to apple and *agrobacterium tumefaciens* a casual agent of Crown Gall disease (Jeffrey *et al*., 2008).

REVIEW OF LITERATURE

Actinomycetes population from continental slope sediment of the Bay of Bengal was studied by Das *et al.,* (2008). The range of actinomycete population is from 5.17 to 51.94 CFU/g and 9.38 to 45.22 CFU/g dry sediment weight. From stations in 1000 m depth no actinomycete colony was isolated. Populations in stations in 500 m depth in both cruises were higher than that of 200 m depth stations. Three actinomycetes genera were identified. Found *Streptomyces* was the dominating one in both the cruises, followed by *Micromonospora,* and *Actinomyces*. Spiral spore chain showed the maximum abundance and the spore surface was smooth.

A study was done by Jeffrey *et al*. (2008) by the collection of 62 isolates of actinomycetes isolated from 7 soil samples collected from Agriculture Research Center Semongok, Sarawak. All the 62 isolates exhibit dark grey, grey, dark brown, brownish, whitish and yellowish white colours. After that all isolates were purified and a few enzymatic screening was done. The results indicate that, 48, 46 and 41 isolates showed the ability to secrete the enzyme cellulase, lipase and protease respectively. By the selection of phytopathogens as test strains antimicrobial test was done and it was observed that 3, 25, 35 and 37 of the isolates showed antagonistic reaction with *Fusarium palmivora*, *Bacillus subtilis*, *Pantoae dipersa* and *Ralstonia solanacearum* respectively. Most promising six isolates were selected and identified by their 16S rRNA sequence. All the six isolates were identified as *Streptomycetes* spp. To see the use of actinomycetes in agriculture industry further study will be done to fully utilize these potential microbes for sustainable agriculture.

A study was done on the taxonomic characterization of novel metabolite- or enzyme-producing actinomycete isolates by Labeda, 1985. It is an extremely important part of any discovery research program. During last seven years there have been no changes in the process of identification of actinomycetes. During the past several years, some improvements are there in some analytical technology. Further

improvements are expected in the future. The application of the criteria described by Lechevalier and Lechevalier in 1980 to the identification of actinomycete isolates has resulted in the Creation of the new genera *Amycolata* (Lechevalier *et al*., 1986], *Amycolatopsis* (Lechevalier *et al*., 1986), *Glycomyces* (Labeda *et al*., 1985), *Saccharothrix (*Labeda. *et al*., 1984) and *Kibdelosporangium* (Shearer *et al*., 1986). Through the use of these chemotaxonomic standards the species that were misplaced in inappropriate genera have also been reclassified to the appropriate genera. Still the species concept in many genera of the Actinomycetales is poorly defined, but chemotaxonomic numerical taxonomic and molecular taxonomic techniques applications will result in clarification of the systematics within these genera and It is also necessary for the patent applications that might be submitted. Over the past few years the criteria for the characterization of actinomycete strains to the genus level have become standard and it include morphological as well as chemotaxonomic properties. In many actinomycete genera species concept is still poorly understood, and an evaluation of strains within these genera by classical, numerical, and molecular taxonomic techniques may be necessary to clarify this situation.

Vellar Estuary was investigated by Dhanasekaran *et al*., 2009 they collected sample as a source of actinomycetes to screen for the production of novel bioactive compounds. There the actinomycetes count was 12 x 10^4 cfu/g of soil. Insignificant variation is shown by the physiochemical characteristics of soils samples in temperature, pH and dissolved phosphate, and total variation is shown by nitrogen and organic matter. Some of the very capable antibiotics producing actinomycetes are isolated from this ecosystem here it strongly inhibit the growth of both Gram positive and Gram-negative bacteria and also yeast like fungi of the 20 actinomycetes isolate. Among them only 4 isolates exhibits the antimicrobial activity and only one shows broad-spectrum activity, and the strain DPTD-5 was further characterized and its identification was done. Earlier studies done by Joe D' Souza and Nelson De Souza (2000) proved that estuarine soils are rich in actinomycetes and they can produce antibiotics. The samples from estuary are reported as very rich habitat for the microbial diversity. After this the cultural and physiological characterization and its

DNA homology suggest that strain the DPTD-5 is very similar to *Streptomyces bikiniensis.*

A study was done by Crawford *et al*., 1993 on the use of selective media, it includes 267 actinomycete strains that were isolated from four rhizosphere-associated and four non-rhizosphere-associated British soils. For isolating diverse group of actinomycetes the organic media which has low nutrient concentrations were found to be very good by avoiding contamination and overgrowth of isolation media by eubacteria and fungi. All isolates grew well at pH range of 6.5 to 8.0 and only some strains were unable to grow at pH 6.0. A significant number of isolated strains failed to grow at pH 5.5. By the use of a Difco cornmeal agar assay procedure eighty-two isolates were screened through the in vitro antagonism towards *Pythium ultimum*. Among all of them five isolates were very strong antagonists of the fungus, four were strong antagonists, and ten others were weakly antagonistic.

Soil sample was collected by Lakshmipathy and Kannabiran 2010 from the coastal region of Tamil Nadu with the aim of isolating actinobacteria and screen them for antagonistic activity against common bacterial and fungal pathogens. Serial dilution of the soil sample was done and after that subsequent screening of the isolates obtained is resulted in the identification of a potential strain VITDDK2 with significant activity against *Klebsiella pneumoniae*, *Aspergillus flavus* and *Aspergillus niger.* The strain VITDDK2 shows chitinolytic activity. By the Chemotaxonomic analysis the isolate VITDDK2 belongs to cell wall Type I.16 S rRNA partial gene sequence and phylogenetic analysis showed that the strain VITDDK2 shared 93% similarity with *Streptomyces sp.* strain 346.

A Study was done by Nathan *et al*., 2004 which suggested a unique selective enrichment procedure which resulted in the identification and isolation of two new genera which are marine-derived actinobacteria. By this study it is revealed that approximately 90% of the microorganisms were cultured by using the presented method which is from the prospective new genera, it indicates as a result which is indicative of its high selectivity. From the Bismarck Sea and the Solomon Sea off the coast of Papua New Guinea 102 actinomycetes were isolated from the subtidal

marine sediment. By performing the test for physiological and chemotaxonomic characteristics and with this distinguishing 16S rRNA gene sequences, and phylogenetic analysis based on 16S rRNA genes it ultimately provides strong evidence for the two new genera (represented by strains of the PNG1 clade and strain UMM518) within the family *Micromonosporaceae*. Biological activity testing of fermentation products from the new marine-derived actinomycetes showed that ist has activities against multidrug-resistant gram-positive pathogens, malignant cells, and vaccinia virus replication.

Cellulose utilization:

A study was done by Arora *et al.*, 2005 on actinomycete strain Streptomyces griseus B1, isolated from soil, study shows that when it is grown on cellulose powder as submerged culture it produces high levels of all the three components i.e. filter paper lyase (FPase), CMCellulase and bglucosidase of the cellulolytic enzyme system. Extracellular activity is shown by FP activity and CMCelulase and b-glucosidase was both intra- and extra-cellular. When grown on hardwood powder under submerged cultureit shows highest FPase activity. It was not able to use lignin monomers (ferulic acid, vanillic acid and syringic acid) as carbon source. When it grows on hardwood and softwood powders under solid-state conditions, it depletes the cellulose (36.3% in the case of softwood and 14.4% in the case of hardwood). And it also causes partial loss of lignin content in both the substrates by solubilizing them.

A study was done by Arunachalam *et al.,* 2010 on the identification of the cellulase producing actinomycetes from soil of Southwest ghats, Tamilnadu, India. These characterization was done by the morphology, cultural, physiological and chemo taxonomical analysis and phylogenetic analysis of TBG-V20. By the morphological, isolate TBG-V20 was identified as a representative of the genus *Streptomyces* and the 16SrRNA region of this strain was constructed. The carboxy Methyl Cellulose (CMCase) activities of the strain TBG-V20 shown on eighth day and an amount of 935 UL^{-1} of glucose 225µg mL^{-1} of protein and 870 mg/100mL of growth were recorded on tenth day. On first day strainTBG-V20 shows exocellulase activity

and produced an amount of 500 µg mL^{-1} glucose. By seeing the observation and phylogenetic analysis, the strain TBG -V20 exhibits similarity to the species *Streptomyces noboritoensis* and its cellulose production is also determined.

Bioactive compounds

A study was done by Baskaran *et al.,* 2011 various pre-treatment methods and three different media were employed for the isolation of bioactive actinomycetes from mangrove sediments of Andaman and Nicobar Islands, India. Collection of sediment sample was done from four different sites of mangrove forest and pre-treated by dry heat method, after that the media were supplemented with cycloheximide 80 µg/mL and nalidixic acid 75 µg/mL. The mean actinomycetes population density in sediment samples were recorded as 22 CFU-10^{-6}/gm in KUA medium followed by 12 CFU-10^{-6}/gm in AIA medium and 8 CFU-10^{-6}/gm in SCA medium, 42 actinomycetes strain were isolated. All the isolates were evaluated for their antibacterial activity against pathogenic bacteria on two different media. Among all the tested isolates, antibacterial metabolite production was shown by 22 species. They were tested against test bacteria namely, *Staphylococcus aureus, Bacillus subtilis, Salmonella typhi* and *Klebsiella pneumoniae*. Significant inhibition towards the growth of all bacteria which were shown by the actinomycete strains such as A101, A102, A107, A116, A121, A125, A130, F101, F102, F104, F106, De101 and De102. Among all these strains, A107 was identified as *Streptomyces* spp. This strain had shown the maximum activity against all used pathogens on both medium.

Several antibiotics were tested by Williams and Davies, 1965 against a range of actinomycetes, bacteria and fungi representing types found in soil. From these tests four antibiotics, nystatin *(50* pg. /ml.), actidione *(50* pg./ml.), polymyxin B sulphate *(5.0* pg./ml.) and sodium penicillin (1.0 ug./ml.), were selected for incorporation into a starch+casein medium to achieve selective growth of actinomycetes on soil dilution plates. For selective development of actinomycete colonies mixture of antibiotics was tested with a number of soils and its efficiency compared with several other methods. Among all the seven antibiotics tested against

a range of actinomycetes, the antifungal ones (nystatin, actidione) did not inhibit any strains not even at the highest concentration of l00 ug/ml. same type of results were obtained by Okami *et al.* (1959) and by Porter *et al.* (1960). Polymyxin B sulphate has shown the least inhibition by antibacterial antibiotics. Most appropriate mixture for the enumeration of soil actinomycete colonies was starch + casein medium with the two antifungal antibiotics (nystatin, actidione). And for isolation of actinomycete colonies the use of same medium with all four antibiotics was most satisfactory.

A study was done by Pugazhvendan *et al.,* 2010 on marine actinomycetes. They collected sample from Chennai (Tamil Nadu) coastal area. 34 strains were isolated and among them 10 potential marine actinomycetes strains were screened by cross streak method against five fish pathogenic bacteria. The extract was tested by disc diffusion method against fish bacterial pathogens and the ethyl acetate extract showed a good inhibition range of 6-15 mm in diameter. The most potential actinomycete strain was characterized and identified as *Streptomyces* spp.

Actinobacteria producing bioactive compounds were isolated by Kumar *et al* by the serial dilution method fom marine sediments collected from Bay of Bengal at a depth of 10-40m near pudimadaka coast of Andhra pradesh. During the study total 78 isolates were obtained and among all the isolates *Streptomycetes* is predominant. Among all the 78 isolates antibacterial and antifungal activity exhibited by 22 isolates exhibited antibacterial and antifungal activity, respectively. Promising activities was shown by the active isolates BTS-112, BTS-314 and BTs-401. After this the strains were further characterized and identified to be belonging to the genus *Rhodococcus* and *Streptomyces*(Kumar *et al.*, 2011).

A total of 55 actinomycetes isolates from soil sample of Karanjal region in Sundarbans were characterized by Arifuzzaman *et al.,* 2010 for morphological identification and antimicrobial activity. The total numbers of isolates were 27, 14, 11 and 3 which belong to *Actinomyces*, *Nocardia*, *Streptomyces* and *Micromonospora* respectively, as they were identified from the sample. Against one or more gram-

negative pathogenic bacteria such as *Shigella boydii*, *Shigella flexneri*-AN-31153, *Shigella sonnei*, *Pseudomonas*, *Shigella dysenterriae* type-1, *Vibrio cholerae*-0139, *Salmonella typhi*-Ao-12014, *Plesiomonas*, *Hafnia* spp., *Vibrio cholerae*-OGET, and *Escherichia coli*- 186LT twenty actinomycetes isolates were found which can produces antibiotic. A diverse group of actinomycetes were found in sundarbans soil and among them three of the tested isolates had a broader spectrum antibacterial activity that shows there potential as a source of antibiotics for pharmaceutical interest.

A study was done by the Santhi *et al.,* 2010 by the collection of total of two marine actinomycetes isolated from different locations of the Manakudi Estuary of Arabian Sea in Tamilnadu, India. All the isolated strains exhibits higher antagonistic activity against the Gram positive bacteria; methicillin resistant and susceptible *Staphylococcus aureus*, *Enterobacter* sp, *Salmonella typhi, Bacillus subtilis, Klebsiella pneumoniae*, *Proteus vulgaris*. Intermediate activity was shown by them against Gram negative organism *Pseudomas auregionosa and* it shows no antagonistic effect towards yeast like *Candida albicans*. Pink actinomycetes (PJS) with white aerial mycelium and pink substrate mycelium and black colonies (BJS) of white aerial mycelium and yellowish white substrate mycelium shows potent inhibiting effect of other microorganisms. Biochemical analysis of PJS and BJS revealed these organisms are Gram positive and shows negative result for starch, mannose, glucose, sucrose, fructose, lipase and urease positive. All strains were resistant to nalidixic acid, methicillin and penicillin. And after that 16S rDNA phylogenetic typing gave ~1500 bp amplified product and it was cloned in pGEMT easy vector. Sequencing of amplified product will give the phylogeny of isolated actinomycetes and the further study on this organism may provide a new Antibiotic for the welfare of human being .

A total of 94 actinomycete strains were isolated by the You *et al.,* 2005 from the marine sediments of a shrimp farm, 87.2% belonged to the genus Streptomyces, others were *Micromonospora* spp. Among them fifty-one percent of the actinomycete

strains showed activity against the pathogenic *Vibrio* spp. strains. Thirty-eight percent of marine Streptomyces strains produced siderophores on chrome azurol S (CAS) agar plates. From the total strains seven strains of Streptomyces were found to produce siderophores and they inhibit the growth of *Vibrio* spp. in vitro. Two strains are belonged to the Cinerogriseus group, which is the the most frequently isolated group of Streptomyces. From the obtained results it can be assumed that in aquaculture it can be use as biocontrol agent.

A study was done by Imada 2005 on several enzyme-inhibitor-producing actinomycetes isolated from various samples collected from the marine environment and characterized. Among them it is found that they can produce novel compounds which are useful in medicine and agriculture. From neritic sea water a strain of actinomycetes was isolated and characterized which produces antibiotics against gram positive bacteria only in the presence of sea water. And the production of antibiotics was observed at seawater concentrations ranging from 60 to 110% (v/v). Hence, the production was seawater-dependent. They show the production of tetrodotoxin (TTX), it is known otherwise as puffer fish toxin, and it was investigated in various actinomycetes collected from the marine environment. Among the 10 isolates from various sea areas, 9 produced TTX as judged by their retention times on high performance liquid chromatography (HPLC).

A study was done by Thenmozhi and Kannabiran 2010 and the aim of the study was to screen the antifungal activity of the crude extract prepared from the strain *Streptomyces* spp. From the Puducherry coast of India 8 strains were isolated from the marine sediment. Primarily eight strains were screened for antifungal activity against three species of *Aspergillus* namely *A. fumigatus*, *A. niger* and *A. flavus*. This search resulted in the isolation of a potential strain VITSTK7. The optimization was done by the production media for the maximum yield of secondary metabolites. And the metabolites were extracted using ethyl acetate, it is than lyophilized and screened for antifungal activity against the three *Aspergillus* species by well diffusion method. Maximum zone of inhibition observed was 21mm for *A. fumigatus* in comparison with the standard antifungal antibiotic Nystatin which shows 20 mm. By using the Hideo

Nonomura classification the strain was further identified. To identify upto the species level a phylogenetic tree was constructed by maximum parsimony method. The molecular taxonomy and phylogeny revealed that the strain belonged to the genus *Streptomyces* and was designated as *Streptomyces* spp.VITSTK7. After this the blast search of the 16s rRNA sequence of the strain with the sequences available in the NCBI data bank exhibits a maximum similarity of 86% with *Streptomyces longisporoflavus* (DQ 442520) with the bootstrap value of 100. The 16s rRNA sequence of the strain *Streptomyces* spp.VITSTK7 was submitted to the GenBank under the accession number GQ 499369.

Seventy-nine Actinomycetes were isolated from soils of Kalapatthar (5545m), Mount Everest region by Gurung *et al*., 2009. Among all the isolates twenty seven (34.18%) of the isolates showed an antibacterial activity against at least one test-bacteria among two Gram positive and nine Gram negative bacteria in primary screening by the technique of perpendicular streak method. In secondary screening thirteen (48.15%) showed antibacterial activity. After that the MIC test was done and the minimum inhibitory concentration (MIC) of antibacterial metabolites of the isolate K.6.3 was 1mg/ml, and that of isolates K.14.2 and K.58.5 was 2mg/ml. From each of the metabolites two spots were detected on thin layer chromatography plate which was completely different from the spot produced by vancomycin. And the active isolates from primary screening were heterogeneous in their overall macroscopic, biochemical, and physiological characteristics.

OBJECTIVE

- Collection of sediment sample from Bhitarkaniaka mangroves forest.
- Isolation of pure culture of Actinomycetes from wet and dry sample.
- Phenotypic characterization of cultered sample of actinomycetaes.
- Biochemical characterization of actinomycetes.
- Antimicrobial testing of all strains of actinomycetes.
- Enzymatic screening of actinomycetes.

MATERIALS AND METHOD

Sampling:

A study was done by the collection of two soil samples from two different sites of Mangrove Bhitarkanika National park (Figure 2 and 3).

Study area:

Orissa coast is mainly depositional in nature, form by the Mahanadi and the Brahmani- Baitarani deltas. The State has a long coastline of 480 kms, lagoons and offshore islands on the eastern part are distributed along the Bay of Bengal. The coastal plains of the State are spreaded from Subarnarekha River in the north to the Rushikulya in the south and are also narrow in the north. Subarnarekha and Mahanadi are the major estuaries and Rushikulya, Bahuda, Devi, Balijhori, Ghalia, Kharnasi, Jambu,etc are the minor estuaries. In asia Chilka Lake is the largest brackish water lagoon and stretches over an area of 1100km. Bhitarkanika is known as the second largest mangrove ecosystem of India, it comes next to Sundarbans mangroves. Gahirmatha is the largest breeding ground for Olive Ridley. Geographically Bhitarkanika is located between 20°4'-20°8'N Latitudes and 86°45'-87° 50' Longitudes .as it is the second largest mangrove ecosystem of India, it has very significance in regard to ecological, geomorphologic and biological background that includes mangrove forests, creeks, rivers, estuaries, accreted, backwater land and mud flats. Bhitarkanika mangrove ecosystem flourishes in the deltaic region, formed by the rich alluvial deposits of Brahmani, Baitarani and the Dhamra River.

The Bhitarkanika Mangroves are a mangrove wetland located in north-eastern corner of Kendrapara district of India's Orissa State. It presents a salt tolerant, complex and a very dynamic eco-system that occurs only in tropical and subtropical inter-tidal areas. The rich, lush green, vibrant eco-system lies in the estuarine region of Brahmani and Baitarani. Comprising mangrove forests, rivers, creeks, estuaries, back water, accreted land and mud flats, Bhitarkanika is significant for its unique

ecological, geomorphological and biological profile that has evolved over centuries to its present status. The Bhitarkanika Mangroves forest covers an area of 650 sq. km and it comes in one of India's largest populations of saltwater crocodiles (*Crocodylus porosus*). The Gahirmatha Beach separates the mangroves from the Bay of Bengal, is the world's most important nesting beach for Olive Ridley Sea Turtles (*Lepidochelys olivacea*). The wetland also hosts a large and diverse population of resident and migratory birds from Central Asia and Europe,that congregate in Bagagahan heronry, an area of approximately 4 hectares within the Bhitarkanika Forest Block near Suajore creek during June to October every year. Bhitarkanika also houses endangered wildlife like Indian python, King cobra, black ibis, spotted dear, Sambar, Wild boar, Jungle cat, fishing cat and darters. It is the best reptile refuge in the country (Mohanty, 1992).

Location of sample collection:-

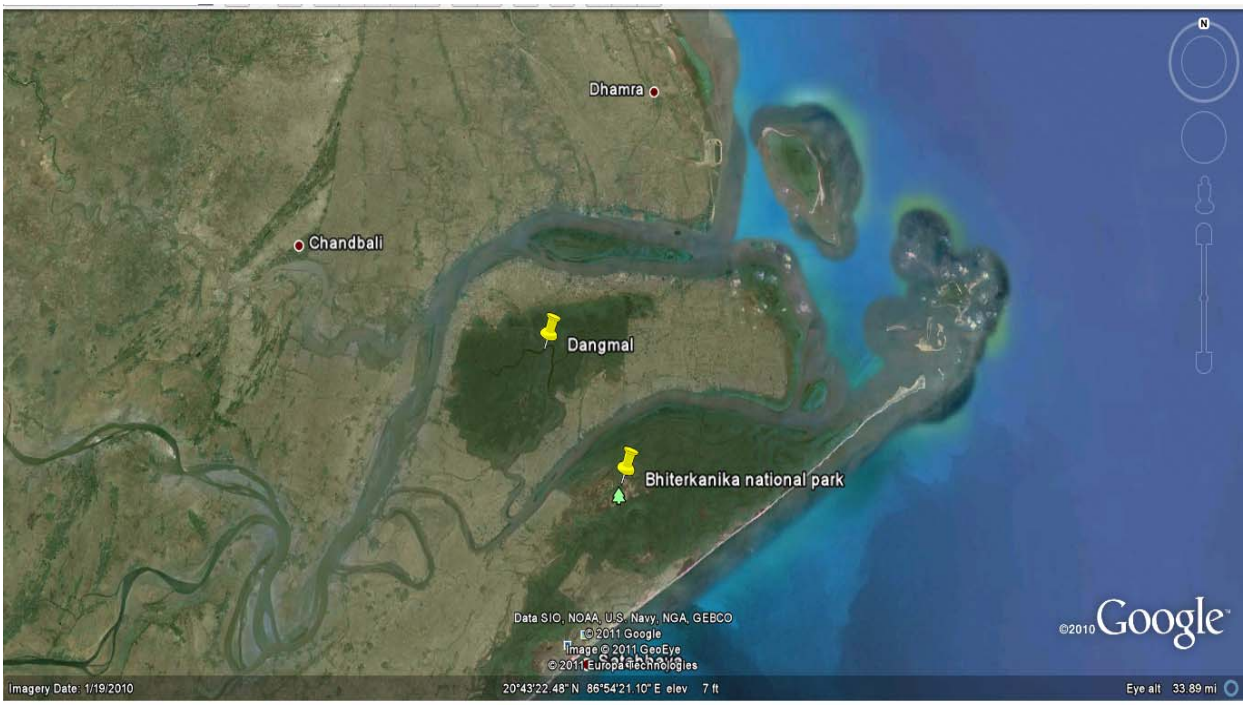


Figure 2. Overview of mangrove ecosystem at Bhitarkanika

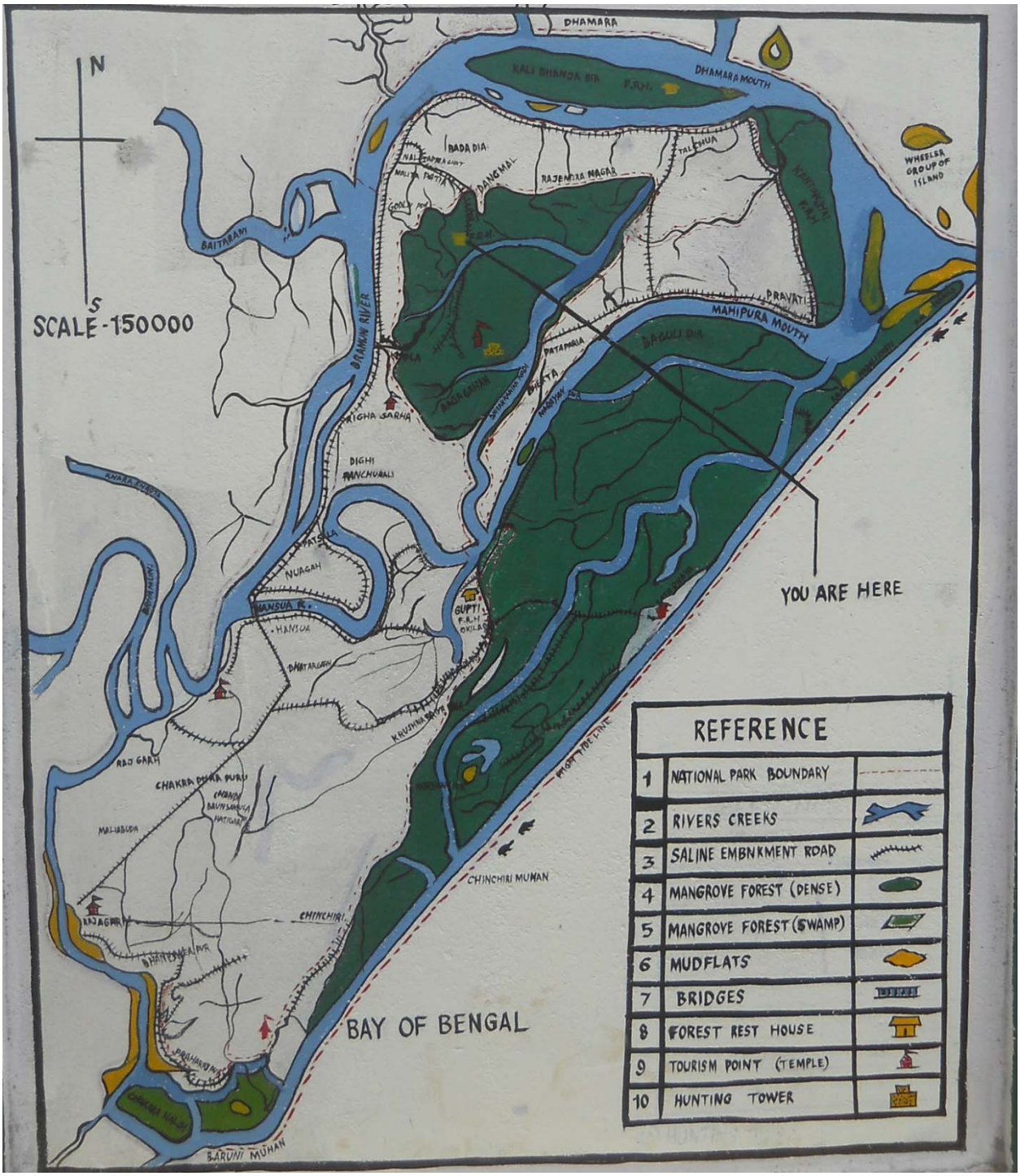


Figure 3. Site Map of Mangrove ecosystem of Bhitarkanika, Odisha

Table no: 1

Geographical position of sampling location

District	Site	Ecological importance	Geographic location	Area in km^2
Kendrapara	Bhitarkanika	Mangroves, Breeding and spawning ground of reptiles	20° 4'-20° 8N 86° 45'-8750'E	267.14

Sample collection:

The samples were collected in the month of February from top 4 cm soil profile where most of the microbial activity takes place, and thus where most of the bacterial population is concentrated. Soil sample (approx. 5oog) were collected by using clean, dry and sterile polythene bags along with sterial spatula, marking pen, rubber band and other accessories. The site selection was done by taking care of the point where widely varying characteristics as possible with regard to the organic matter, moisture content, and particle size and colour of soil and to avoid contamination as far as possible. Samples were stored in iceboxes and transported to the laboratory where they were kept in refrigerator at 4^0C until analysis.

Isolation of actinomycetes from soil sample:

Each sediment sample is divided in to two parts. At first one part is used as wet sample and the second part was taken inside dryer for one week to be used as dry sample, this helps in decreasing the population of gram negative bacteria. The samples were taken for the serial dilution upto the 10^3 dilution, 0.2 ml of each dilution were inoculated in duplicate plates of the ISP2 media for the isolation of actinomycetes by the spread plate technique. After incubation all plates incubated at 37^oC in the incubator for 7 days. Both the samples were processed as wet and dry sample. By the pure culture technique strains of actinomycetes were isolated. Nystatin and nalidixic acid were used as antifungal and antimicrobial agent respectively in plates. 15 pure strains of actinomycetes have been isolated by streak

plate method. Strains were identified on the basis of their Phenotypic characterizations and Physiological and biochemical characteristics and 16S rRNA.

1. Phenotypic characterizations:

The classification of actinomycete was originally based largely upon the morphological observations. So,morphology is still an important characteristic for the description of taxa and it is not adequate in itself to differentiate between many genera. In fact, it was the only characteristic which was used in many early descriptions, particularly of *Streptomyces* species in the first few editions of Bergey's Manual. These observations are best made by the variety of standard cultivation media. Several of the media suggested for the International *Streptomyces* Project (Shirling and Gottlieb, 1966) and by Pridham *et al*. (1957) have proven to be useful in our hands for the characterization of strains accessioned into the ARS Actinomycetales Culture Collection (Labeda, 1985). It iincludes some basic tests. Aerial mass colour, Reverse side pigment, Melanoid pigments, Spore chain morphology and Spore morphology.

a) Aerial mass colour:

For the grouping and identification of actinomycetes the Chromogenicity of the aerial mycelium is considered to be an important character. The colours of the mature sporulating aerial mycelium are white, gray, red, green, blue and violet following Prauser (1964). When the aerial mass colour falls between two colours series, both the colors are recorded. In the cases where aerial mass colour of a strain showed intermediate tints, then in that place both the colour series should be noted. (Shirling and Gottileb, 1966).

b) **Reverse side pigments*:***

The strains are divided into two groups according to their ability to produce characteristic pigments on the reverse side of the colony, called as distinctive (+) and not distinctive or none (-).A colour with low chroma such as pale yellow, olive or yellowish brown occurs, it is included in the latter group (-).

c) Melanoid Pigments*:*

The grouping is made on the production of melanoid pigments (i.e. greenish brown, brownish black or distinct brown pigment modified by other colours) on the medium. The strains are grouped as melanoid pigment produced (+) and not produced (-) (Shirling and Gottileb, 1966). For the melanoid pigment observation the inoculated plates where kept under incubater for 4 to 5 days. The strains which shows cultures forming a greenish brown to brown to black diffusible pigment or a distinct brown pigment modified by other color are recorded as positive (+) total absence of diffusible pigment, are recorded as negative (-) for melanoid pigment production.

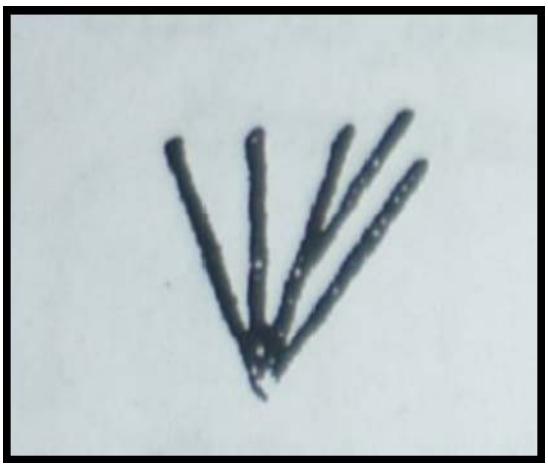
d) Spore chain morphology:

The species with spore bearing hyphae are reported to be three types:-

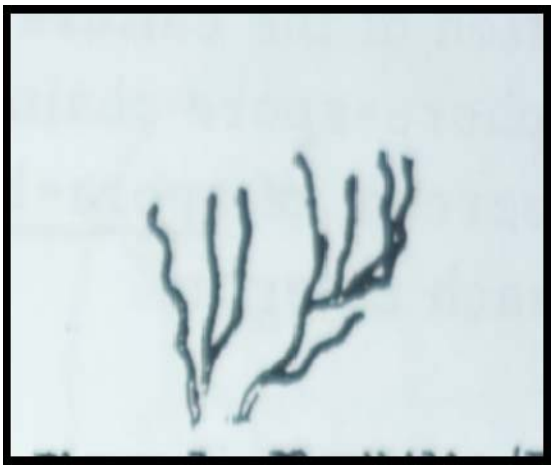
- Flexible- Rectiflexibiles (RF),
- Open loops- Retinaculiaperti (RA)
- Spira- Spirales (S).

A characteristic of the spore bearing hyphae and spore chains is determined by the direct microscopic examination of the culture area. Adequate magnification used to establish the presence or absence of spore chains and to observe the nature of spore chains is 40x. By the standard protocol of cover slip culture technique the plates were prepared and after the incubation of 7 to 10 days it is observed for the

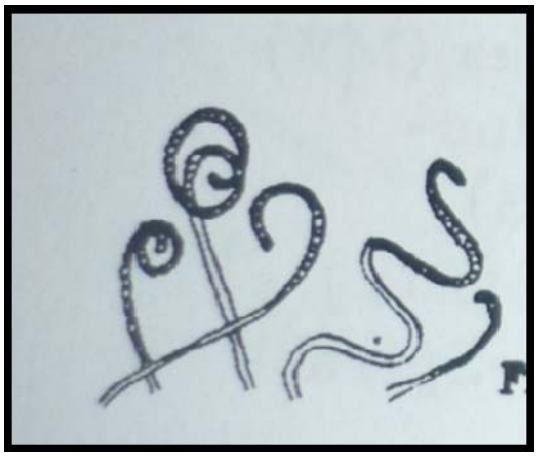
spore chain morphology as shown in , Simple (Fig 4) and verticillate (Fig 5).



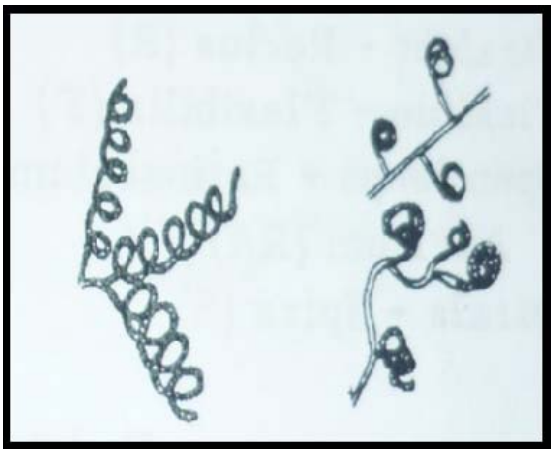
a)Straight- Rectus (R)



b) Flexible- Flexibilis (F)

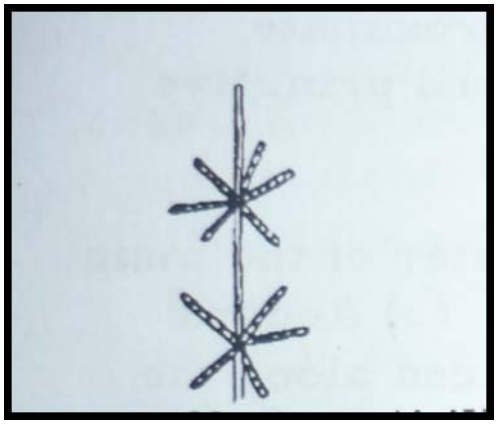


c) Open loops - Retinaculum- Apertum (RA)

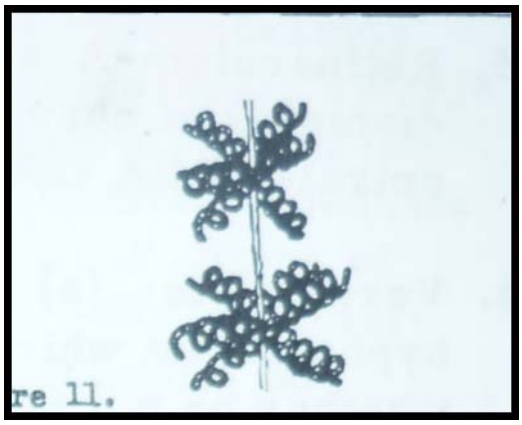


d) Spirals- Spira (S)

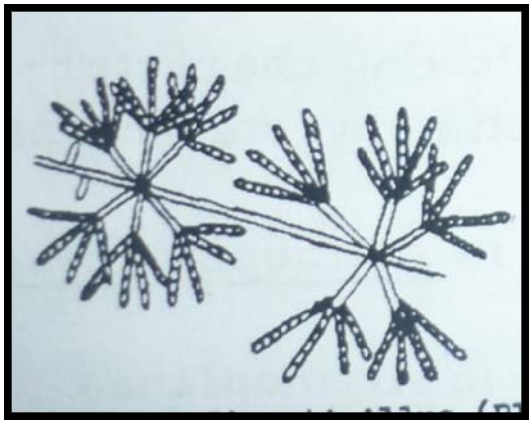
Figure 4 (a – d) Types of spore bearing hyphae



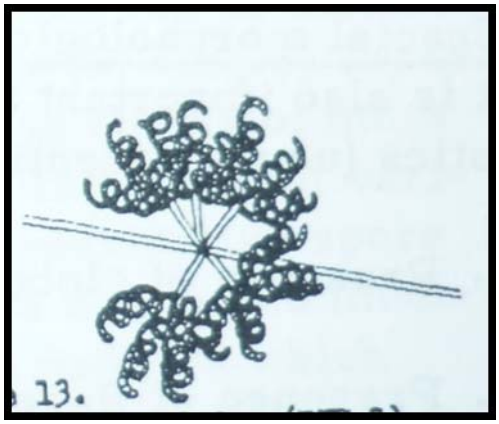
a) Monoverticillus (MV)



b) Monoverticillus - Spira (MV-S)



c) Biverticillus (BIV)



d) Biverticillus-Spira (BIV-S)

Figure 5 (a-d). Types of Spore bearing hyphae

During this method of spore morphological study Plates have to be prepared containing ISP2 medium. After solidification, by a sharp scalpel from the central portion of the plate, medium should be scooped out making a rectangular area. Then three sterile coverslips have to be placed on the hollow rectangular space. Slowly actinomycetes spores have to be inoculated at the edge of the coverslips touching the medium. The plates must be incubated at 28±2°C for 5 days and examined periodically taking out the coverslips.

e) Spore surface morphology*:*

Spore surface features are observed under the scanning electron microscope (SEM). The coverslip culture technique prepared for observation under the light microscope is used for this purpose. The electron grid is cleaned and adhesive tape is placed on the surface of the grid. The spore structures in actinomycetes are reported to be four types39- smooth (sm), spiny (sp), warty (wa) and hairy (ha) (Figure 6).

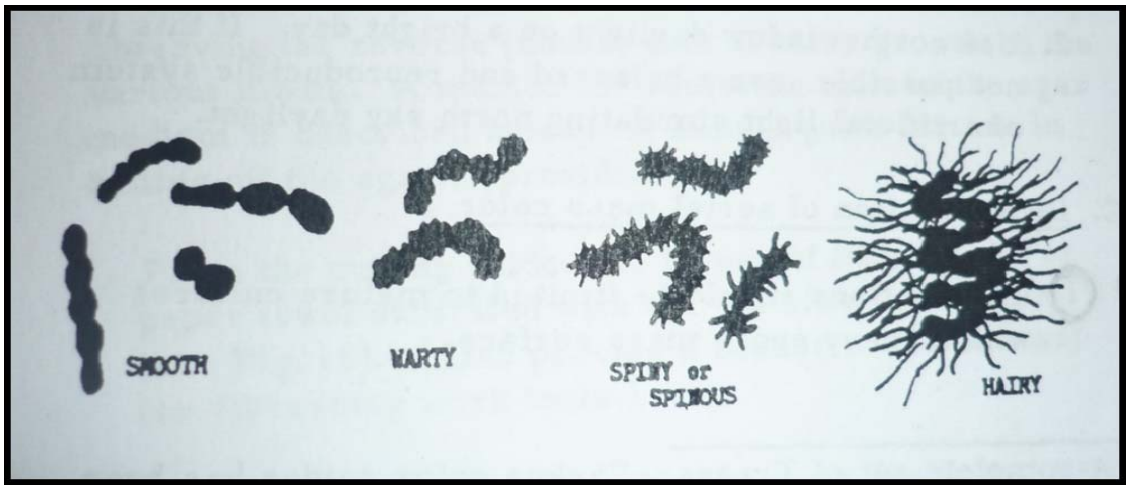


Figure 6 - Spore morphology of actinomycetes

Preparation of culture media:

Table 2: ISP2 Media Compositions

Sl.no	Ingredients	Gms/lit
1.	Yeast extract	4.0 g
2.	Malt extract	10.0 g
3.	Dextrose	4.0 g
4.	Agar	20.0 g
5.	Distilled water	1 lit

pH: 7.3

f) Screening for antimicrobial activity

Screening of Actinomycetes is done by the antimicrobial activity, preliminarily studied by cross streak method against five pathogenic bacteria, the 15 isolated Actinomycetes strains were streaked as parallel line on Nutrient agar plates and incubatedat 28^0C for 5 day. After observing a good ribbon- like growth of the actinomycetes on the petriplates, the pathogen was streaked at right angles to the original streak of actinomycetes and incubated at $28^oC \pm 2^oC$.The inhibition zone was measured after 24 and 48 hr. A control plate was also maintained without inoculating the actinomycetes, to assess the normal growth of the bacteria. Based on the results of antagonistic activity the strains were selected for further studies.

2. Species affiliation- Physiological and biochemical characteristics:

a) Assimilation of carbon sources*:*

The ability of different actinomycetes strains in utilizing various carbon compounds as source of energy was studied by following the method recommended in International Streptomyces Project. Carbon utilization medium (Modified from 'Pridham and Gottlieb, 1948). Stock solution of 10 sugars i.e; D-glucose, L-arabinose, Sucrose , D-fructose, D – xylose, Raffinose, D-mannitol , Cellulose, Rhamnose, inositol having concentration of 10x was prepared in autoclaved water and sterilized by filtering through 0.22µm pore size membrane filters and stored at 4^0C. Growth of actinomycetes strain was checked by taking 1% carbon source in ISP2 media. Plates were streaked by inoculation loop by flame sterilization technique and Incubated at 37^0C for 7 to 10 days. Growths were observed by compairing them with positive and negative control.

A. Sterile carbon sources Use chemically pure carbon sources certified to be free of admixture with other carbohydrates or contaminating materials.

B. Carbon sources for this test are:

- No carbon source (negative control)

- D-glucose (positive control)
- L-arabinose
- Sucrose
- D-fructose
- D - xylose
- I-inositol
- Raffinose
- D-mannitol
- Cellulose
- Rhamnose

These carbon sources were sterilized by membrane filtration without heating. Carbon source sterilized by this method was added to the basal mineral salts agar to give a final concentration of 1%. Stock solution was prepared of 10% and 10ml of this is added to the 100ml of basal medium (Table 3). Pridham and Gottlieb trace salts (only 1 ml of this solution was used per liter of final medium)

Table 3 - Composition of Pridham and Gottlieb trace salts

Sl.no		Gms/lit
1	$CuS0_4 .5H_2O$	0.64g
2	$FeS0_4.7H_2O$	0.11g
3	$MnCl_2.4H_2O$	0.79g
4	$ZnS0_4.7H_2O$	0.15g
5	Distilled water	100.0 ml

It is stored at 3-5^{0}C until required for use.

Complete medium: - Basal medium (Table 4) was sterilized and cooled at room temperature. Sterilized carbon source was aseptically added to a concentration of approximately 1%. Mixture was agitated and poured in 25 ml of medium per dish into 9 cm Petri dishes. Each organism requires 2 Petri dishes with no carbon (as a

negative control) plus duplicate plates for each carbon Source tested. Carbohydrate utilization could be determined by growth on carbon utilization medium (ISP2) supplemented with 1% carbon sources.

Table 4 - Composition of Basal mineral salts agar:-

Sl.no	Ingredients	Gms/lit
1	$(NH_4)_2SO_4$	2.64g
2	KH_2PO_4.anhydrous	2.38g
3	$K_2HPO_4.3H_2O$	5.65g
4	$MgSO_4.7H_2O$	1.00g
5	Agar	15.0g
6	Distilled water	1 lit
7	Pridham and Cottlieb trace salts	1.00 ml

pH.:- 6.8-7.0

Results were recorded as follows:-

- Strongly positive utilization (++), when growth on tested carbon in basal medium was equal to or greater than growth on basal medium plus glucose.
- Positive utilization (+), when growth on tested carbon was simificantly better than on basal medium without carbon, but somewhat less than on glucose.
- Utilization doubtful (±), when growth on tested carbon was only slightly better than on the basal medium without carbon and significantly less than with glucose.
- Utilization negative (-), when growth was similar to or less than growth on basal medium without carbon.

b) Sodium chloride tolerance*:*

Different concentrations of sodium chloride (0, 5, 10, 15, 20, 25 and 35%) solution were added to the starch casein agar medium to check the sodium chloride tolerance test. This test was very much important to understand the native nature of the marine actinomycetes isolates. The isolate was streaked on the agar medium,

incubated at 37°C for 7-15 days and the presence or absence of growth was recorded on 7th day onwards.

c) Degradation of cellulose:

1% of Carboxy methyl cellulose (CMC) was added to the ISP 2 media. The plates were inoculated and incubated for 7-15 days. Control plate was used as standard to check the growth of actinomycetes after 7- 15 days for cellulose degradation activity which may be visually observed.

d) Hydrogen-sulphide production*:*

The inoculated Tryptone-Yeast extract agar slants were incubated for 7 days for this test. Observations on the presence of the characteristic greenish-brown, brown, bluish-black or black colour of the substrate, indicative of H_2S production were recorded on 7th, 10th and 15th days. The incubated tubes were compared with un-inoculated controls.

e) Gelatin liquefaction*:*

Due to the absence of tryptophan Gelatin is called as incomplete protein its value in identifying bacterial species is well established. Gelatine is a protein produced by hydrolysis of collagen, a major component of connective tissue and tendons in humans and other animals. Below temperature of 25^{o}C, gelatine will maintain its gel properties and exist as a solid at temperatures above 25^{o}C, gelatin is liquid. Liquefaction is accomplished by some microorganisms capable of producing a proteolytic extracellular enzyme called gelatinase, which acts to hydrolyze this protein to amino acids. Once this degradation occurs, even very low temperatures of 4^{0}C will not restore the gel characteristic. Gelatin deep tubes were used to demonstrate the hydrolytic activity of gelatinase.

The medium consists of nutrient supplemented with 12% gelatine this high gelatin concentration results in a stiff medium and also serves as the substrate for the activity of gelatinase. Gelatin liquefaction is studied by sub-culturing the strain on gelatin agar medium and inoculated them at 37°C. Observation should be made after

7 days. The extent of liquefaction should be recorded after keeping the tubes in cold conditions (5-10°C) for an hour. Cultures that remain liquefied were indicative of slow gelatin hydrolysis.

Some types of gelatin test slants:-

Liquefaction configurations

- Crateriform: Saucer- shaped liquefaction
- Napiform: turniplike
- Infundibular: Funnel-like or inverted cone
- Saccate: Elongate sac, tubular, cylindrical
- Stratiform: liquefied to the walls of the tube in the upper region

Growth without liquefaction:

- Filiform
- Beaded
- Papillate
- Villous
- Arborescent

f) Hydrolysis of starch*:*

Starch is a high molecular-weight, branching polymer composed of glucose molecule linked together by glycosidic bonds. The degradation of this macromolecule first requires the presence of the extracellular enzyme amylase for its hydrolysis into shorter polysaccharides, namely dextrins,and ultimately into maltose molecules. The final hydrolysis of this disaccharide, which is catalyzed by maltase, yields low molecular weight, soluble glucose molecules that can be transported into the cell and used for energy production through the process of glycolysis.

For this test, cultures should be grown for 5-7 days on ISP2 media. The development of clear zone around the culture streaks, when the plates were flooded with Lugol's iodine solution should be recorded as the hydrolysis of starch. The medium is composed of ISP2 Media supplemented with 1% starch, which serves as the polysaccharide substrate.

g) Coagulation of milk*:*

Milk coagulation was studied with skimmed milk (Hi media). The skimmed milk tubes were inoculated and incubated at 37°C. The extent of coagulation was recorded on the 7th and 10th days of incubation. The importance of milk as a culture medium for the study of bacteria has long been recognized by workers in bacteriology; although milk is a very complex medium, it is more or less standard in composition and the reactions produced upon it by microorganisms are so characteristic, that it has found general acceptance. The plates were incubated for the 7 to 10 days after the inoculation

h) Ability to grow in different pH:

Principle: - pH is defined as logarithm to the base 10 of the inverse of the hydrogen ion concentration (or preferably H^+ ion activity). It is also the negative logarithm to the base 10 of H^+ ion activity. This test was carried out on ISP2 media. pH was adjusted to different ranges of 5, 6, 7, 8, 9 and 10. Duplicate slants were prepared for each strain of each range. After the incubation of 10-12 days readings were taken for each strain.

I) Lipolytic activity

The formation of lipase is demonstrated by adding water-soluble Tweens to a nutrient medium. Around the colonies with lipolytic activity there appears a well visible halo which is due to crystal of the calcium salt of the fatty acid liberated by lipolysis.

There are some advantages of the use of Tween.

- There is an optimum contact between the growing cells and the fatty substrate, which is necessary in those cases in which the formation of lipase is adaptive.
- They can be used in studies on the specificity of the lipase.
- The results are directly visible.

This test was done by taking 1% Tween 20 (Himedia) with ISP2 media. Incubated at the temperature of 37^0C for 7 to 10 days.

Finally, after all these experiments results have been matched with the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project) and the species identification was done.

RESULTS

Sediment sample was inoculated by serial dilution and 15 pure strains were isolated by streak plate method.

1. Phenotypic characterization

Morphology is an important characteristic for the description of taxa, it is not adequate in itself to differentiate between many genera. And in fact, it was the only characteristic used in many early descriptions.

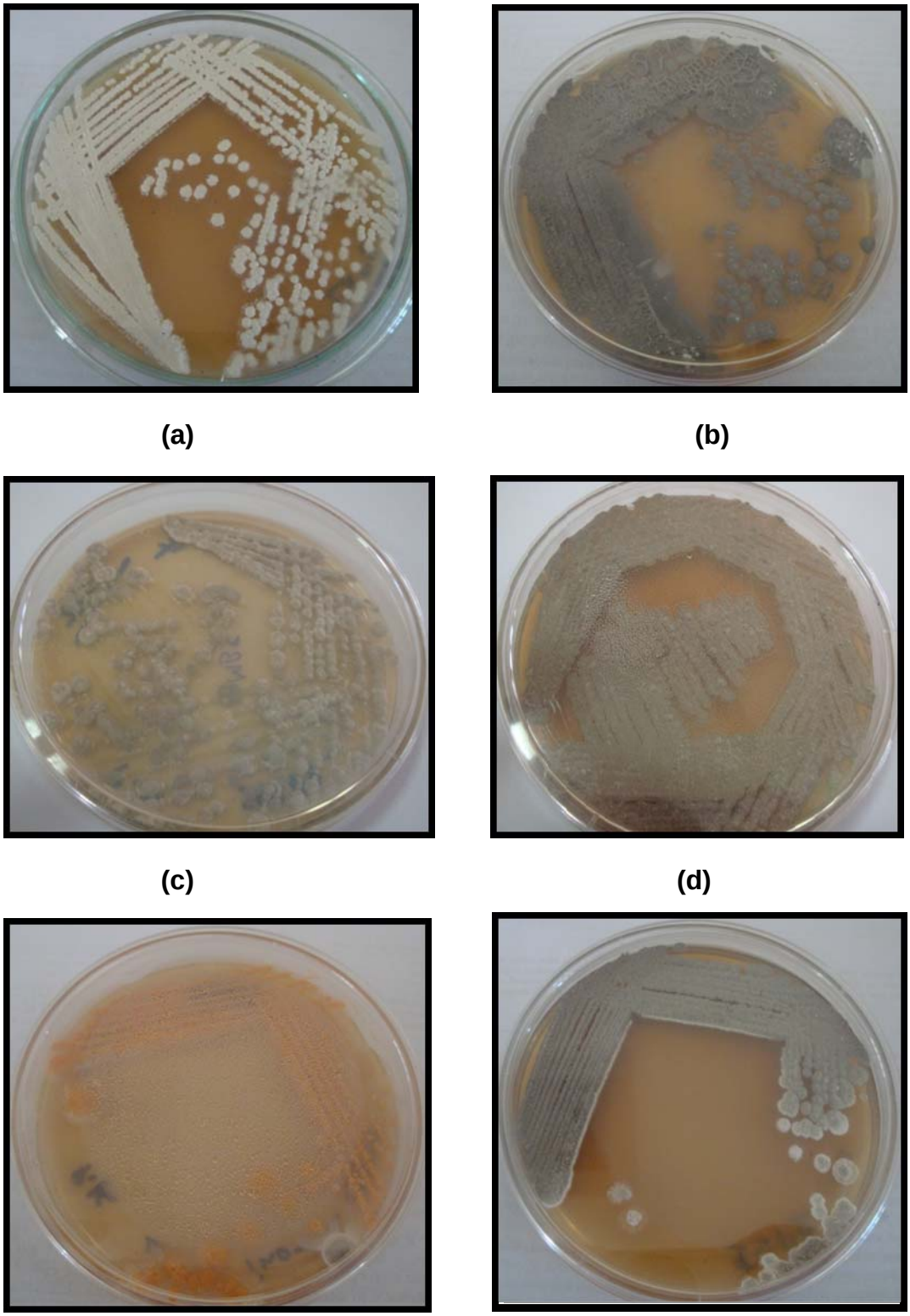
a). Aerial mass colour:-

The colour of the substrate mycelium was determined by observing (Table 5) the plates after 7 to 10 days. It was done only after seeing the heavy spore mass surface.

Table 5 – Readings of Aerial mass colour of actinomycetes.

Sl.no	STRAIN	Aerial mass colour
1	B2	W
2	BS	Gy(W)
3	CO	Gy
4	CS	Gy
5	C1	WGy
6	C8	Gy
7	C9	W(Gy)
8	C11	W
9	C12	GyW
10	D1	Y
11	MB1	W
12	MB2	Gy
13	MB3	WGy
14	MB5	W(Gy)
15	MB9	W

The common colours found in the strains were White and Gray shown in Figure 7.



(a) **(b)**

(c) **(d)**

(e) (f)

Figure 7- (a-e) Aerial mass colour of actinomycetes strains

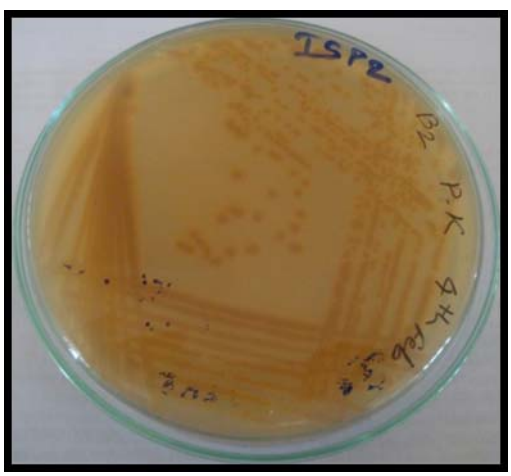
a) Reverse side pigment, Melanoid pigments and soluble pigments

The strains were divided into two groups according to their ability to produce pigments on the reverse side of the colony, namely distinctive (1) and not distinctive or none (0) (Table 6) for Reverse side pigments and Melanoid pigmentation was observed by the formation of greenish brown, brownish black or distinct brown pigment.

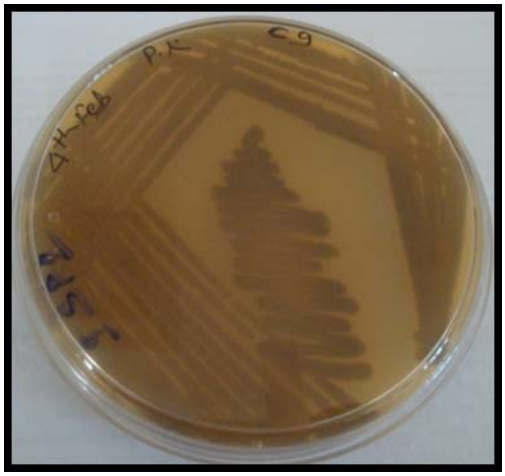
Table 6- Readings of Reverse side pigment of actinomycetes .

Sl.no	Strain	Soluble pigments	Melanoid pigments	Reverse side pigments
1	B2	1	0	0
2	BS	0	0	1
3	CO	1	0	0
4	CS	1	1	0
5	C1	0	1	0
6	C8	0	1	1
7	C9	0	1	1
8	C11	1	0	0
9	C12	1	0	0
10	D1	1	0	0
11	MB1	0	0	0
12	MB2	1	1	0
13	MB3	0	1	1
14	MB5	1	1	0
15	MB9	1	0	0

Colour observed for not distinctive were pale yellow, olive or yellowish brown colour marked as 0 in the Table 6. Most of the strains had shown this formation of pigments. Only four strains namely BS, C8, C9 and MB3 shows the distinctive (1) character shown in Figure 8(a-e). And the melanoid formation was shown in Table no 6. by the presence (1) or absence of (0) of Melanoid pigment here seven strains had shown melanoid formation. Soluble pigment was also observed by seeing the pigmentation of other colours presence (1) and absence (0) six strains had shown negative result.



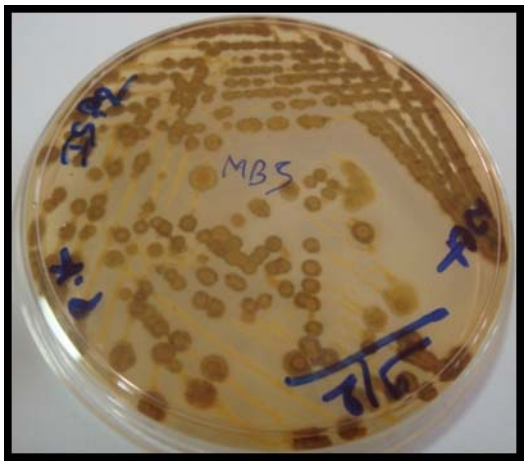
(a)



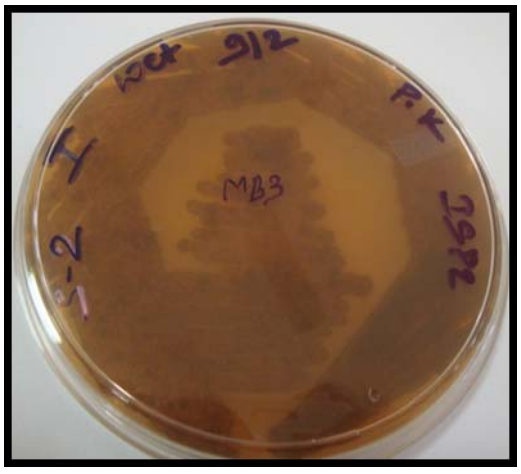
(b)

(c)

(d)



(e)



(f)

Figure 8. (a- e) Showings reverse side pigmentation of actinomycetes strains

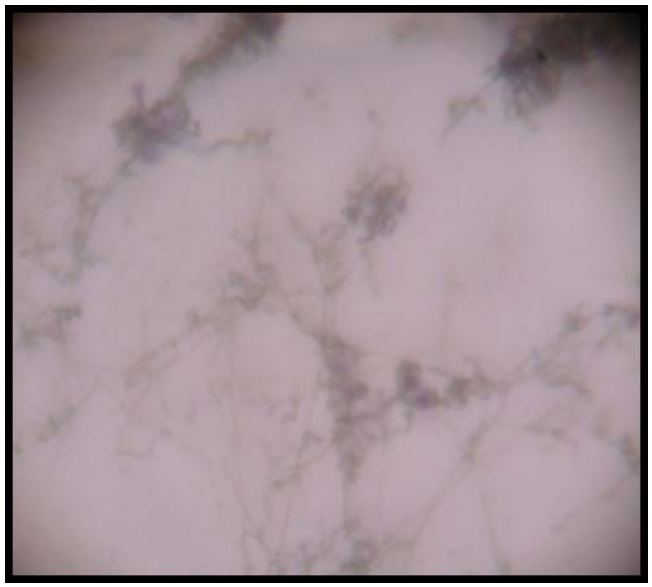
b) Spore chain morphology

This was done by the coverslip culture technique. The slides were examined under microscope of 40x. Spira was shown by maximum strains, only strain BS has shown the simple rectus spore chain and CO has shown Biverticillus - spira spore chain and two strains were shown Retinaculum- Apertum (RA) shown in Table 7.

a) Table 7 Results showing Spore chain morphology

Sl.no	Strain	Spore morphology
1	B2	S
2	BS	R
3	CO	BIV-S
4	CS	S
5	C1	S
6	C8	RA
7	C9	S
8	C11	S
9	C12	S
10	D1	S
11	MB1	S
12	MB2	S
13	MB3	S
14	MB5	RA
15	MB9	RA

All the strains were examined under microscope and accordingly the spore chain morphology was observed shown in from Figure 9 -23.



. Figure. 9 B2

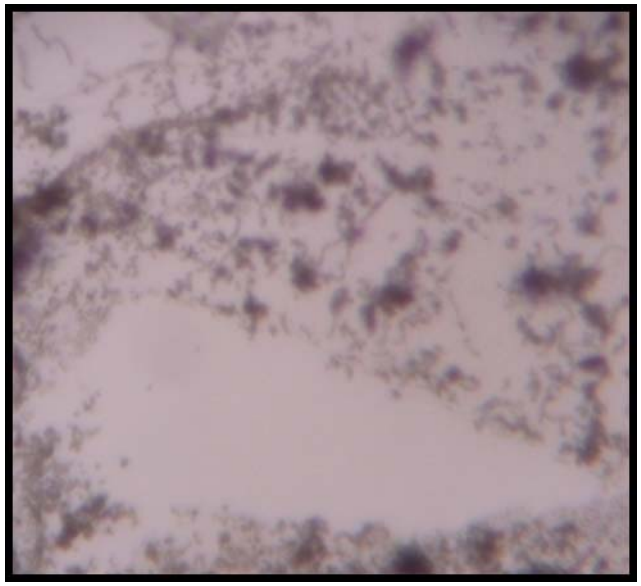


Figure.10 BS

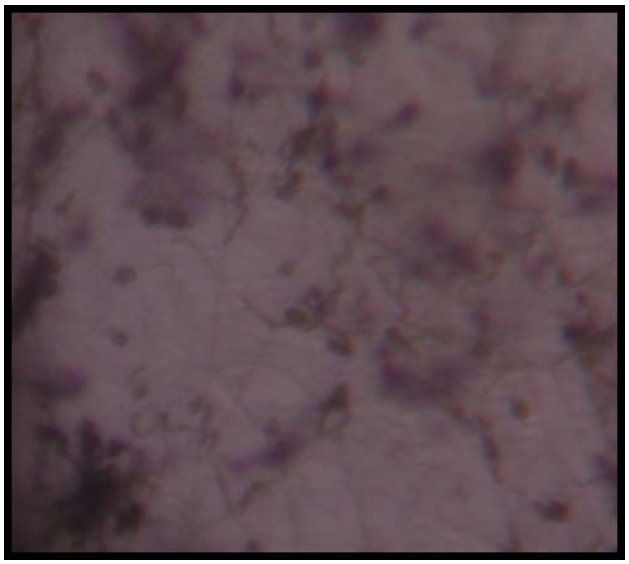


Figure . 11- C1

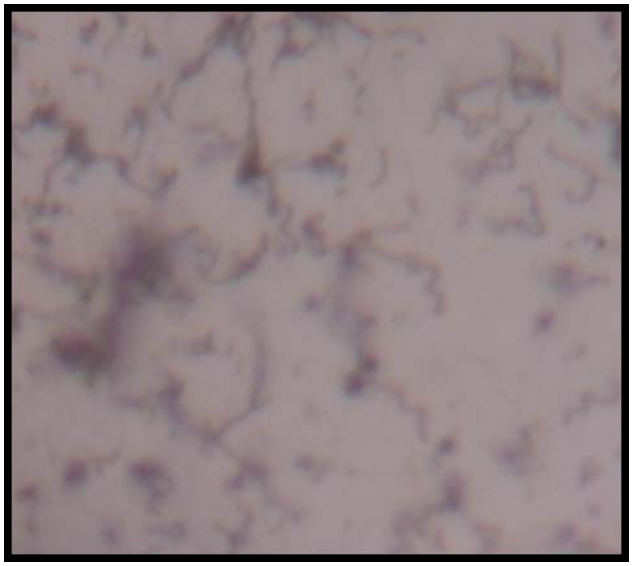


Figure. 12-C8

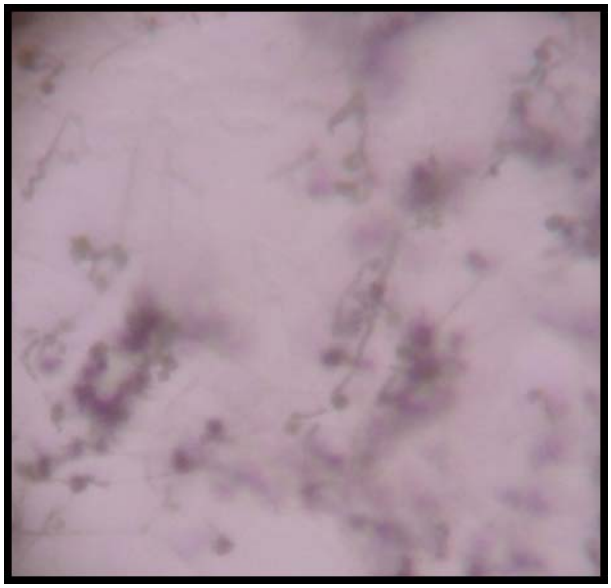


Figure.13- C9

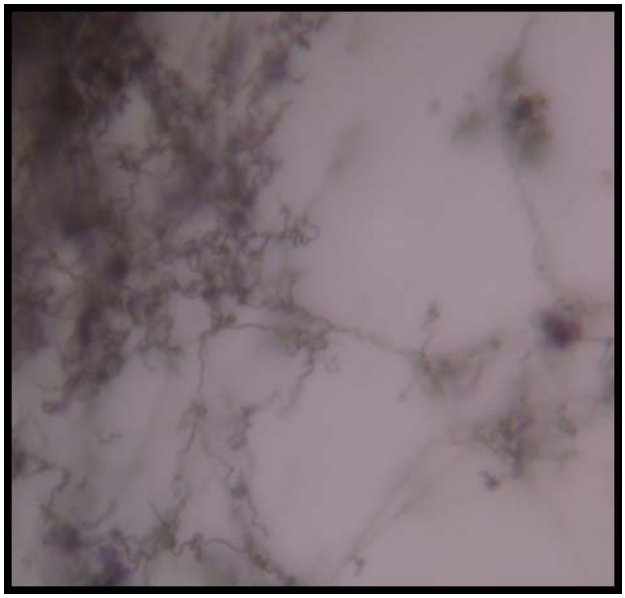


Figure. 14- C11

Figure. 15 CO

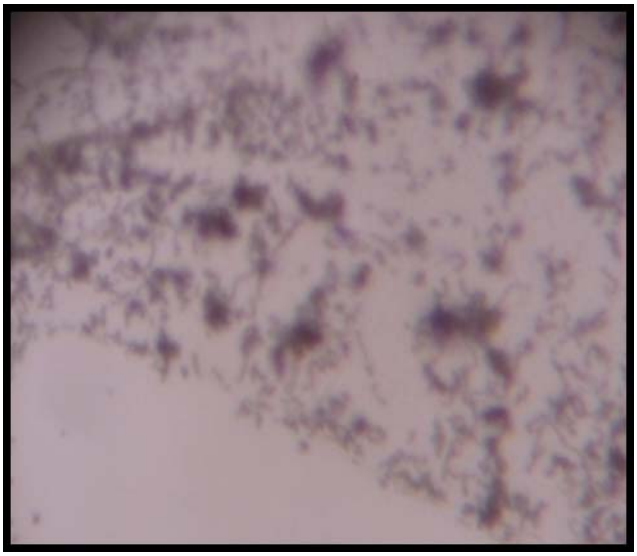


Figure. 16 CS

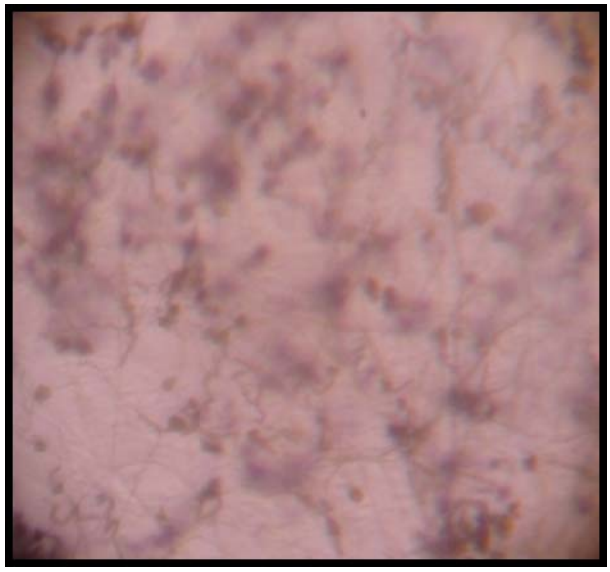


Figure.17 D1

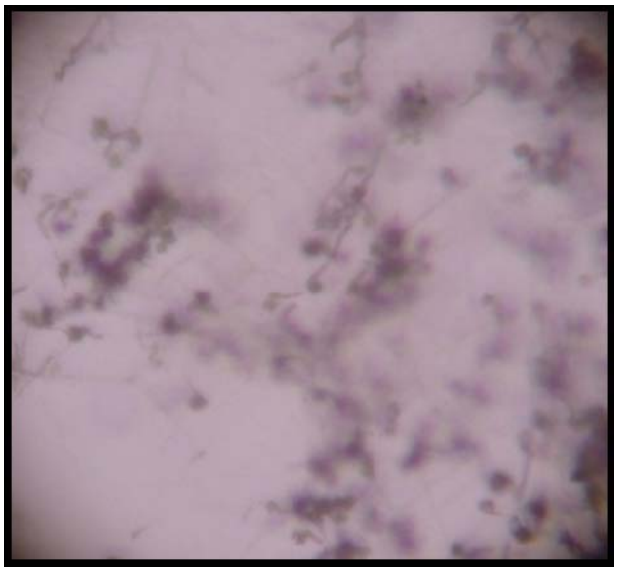


Figure.18 MB1

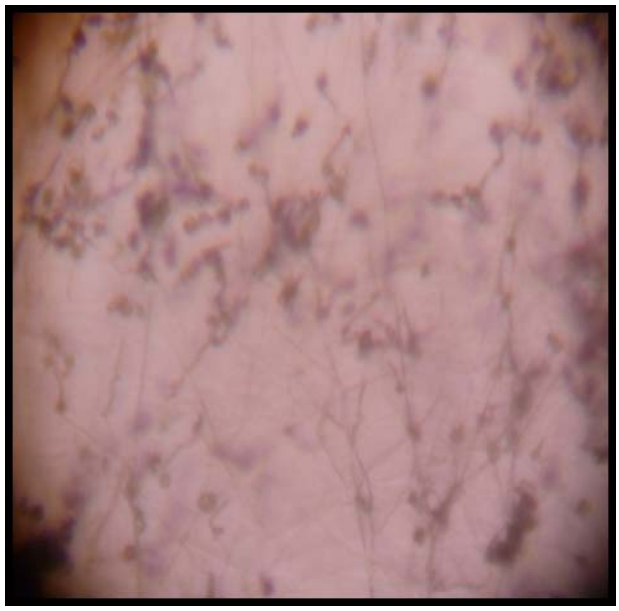


Figure. 19 MB2

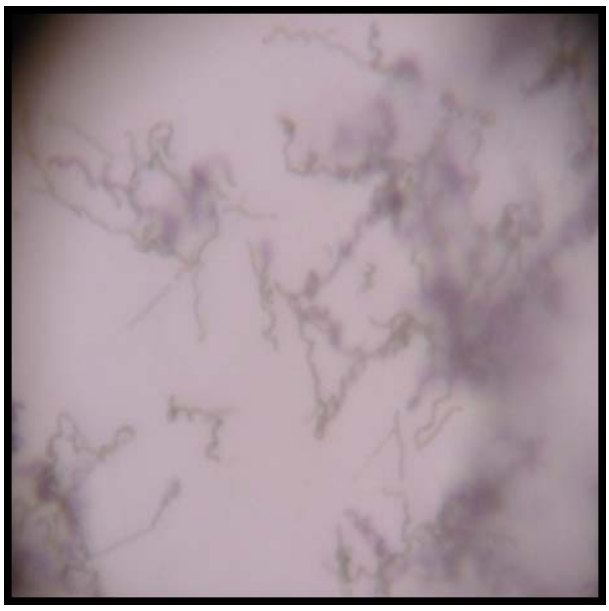


Figure.20 C12

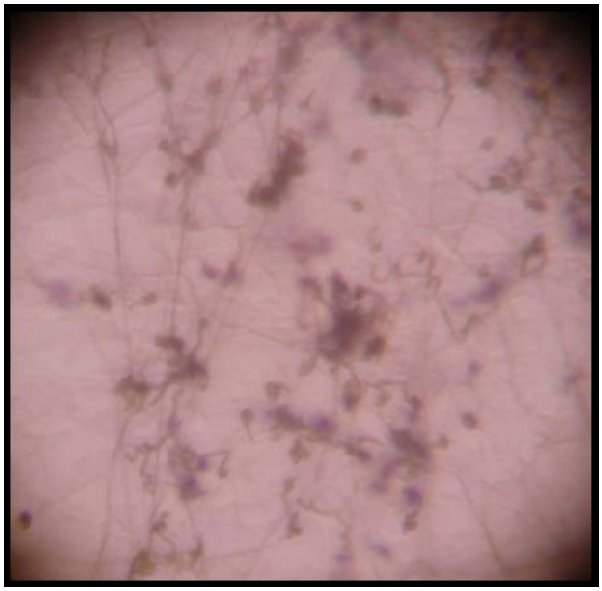


Figure.21 MB3

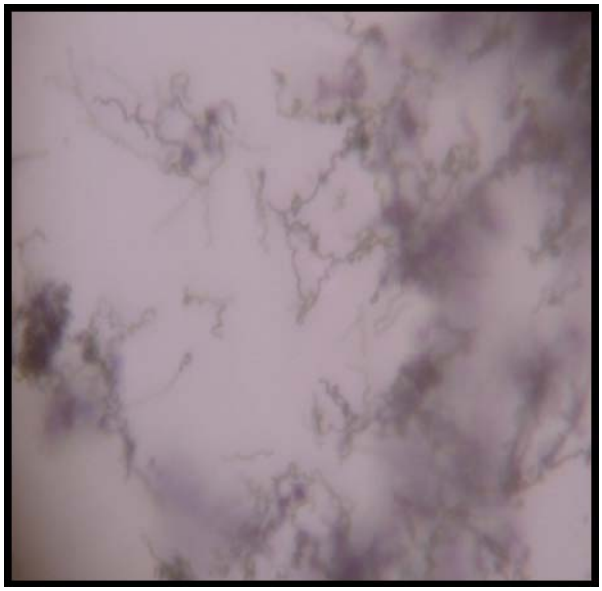


Figure.22 MB5

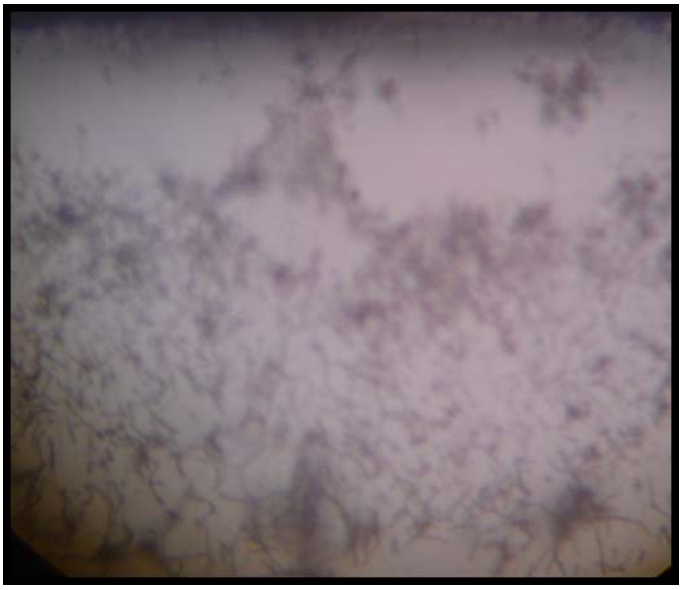


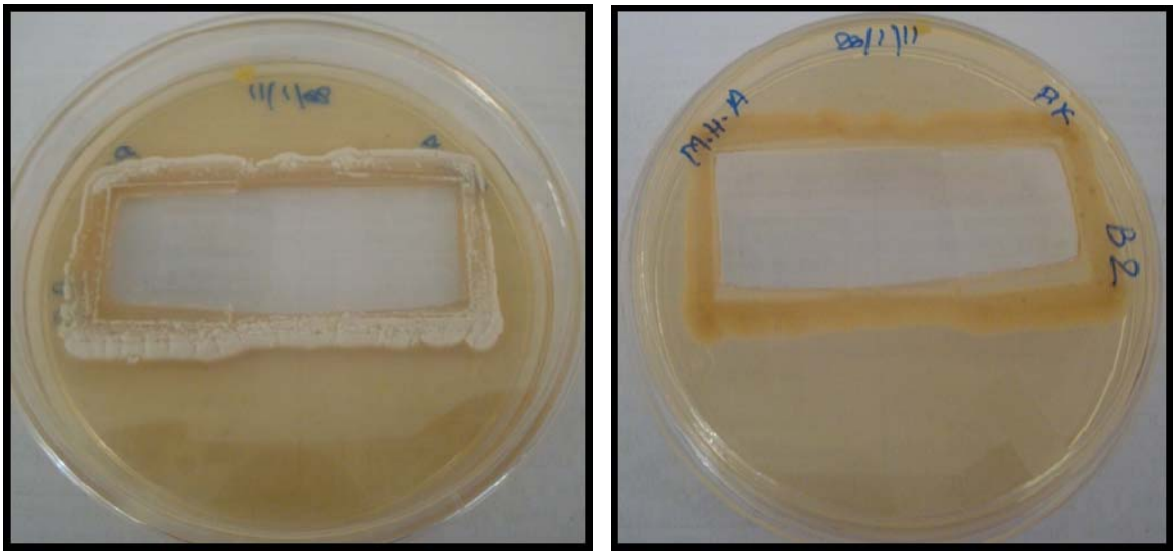
Figure. 23 MB9

c) Spore surface morphology:

Spore surface morphology was studied under the scanning electron microscope (SEM). By the coverslip culture technique (Fig:24 a-b) the slides were prepared of 7 to 10 days incubation. The reading were taken at different magnifications of 500, 3000, 10,000and 20,000. Three strains were seen under SEM shown in Table no 8 and figure from 25 to 27.

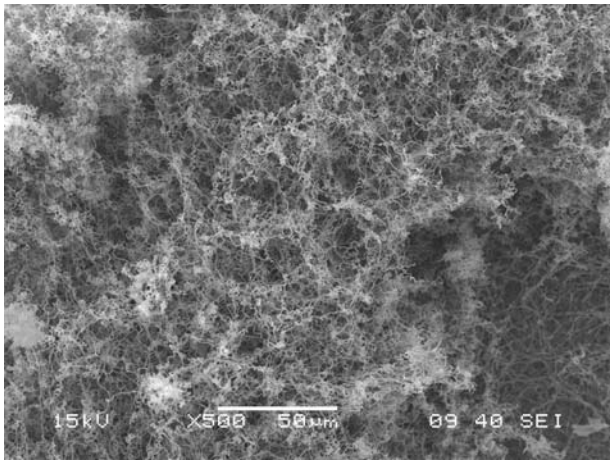
Table no. 8 Readings of actinomycetes by Spore surface morphology

Sl.no	Strain	Spore surface morphology
1.	B2	Smooth
2.	C11	Smooth
3.	CS	Spiny

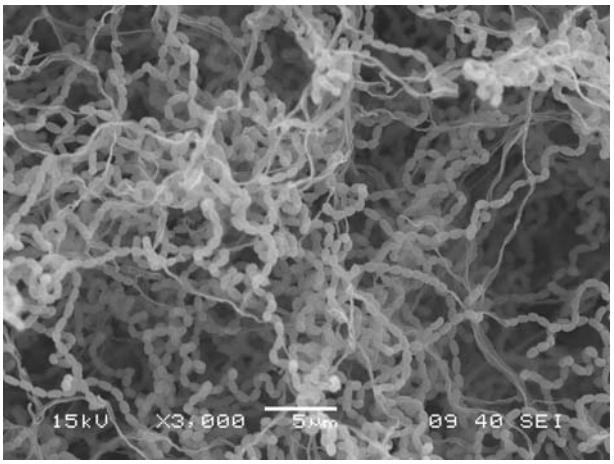


(a) (b)

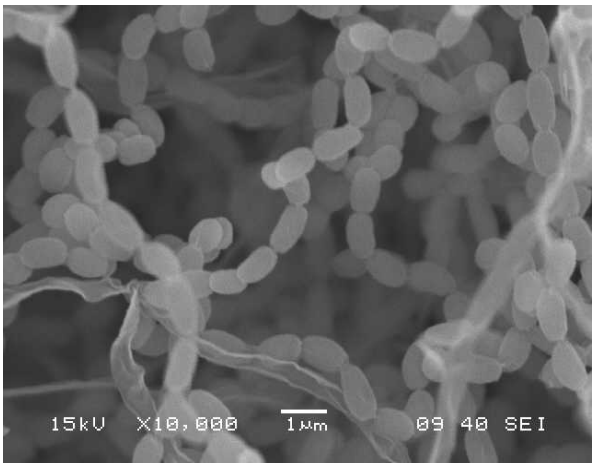
Figure 24 (a-b). Plates showing the Coverslip culture technique



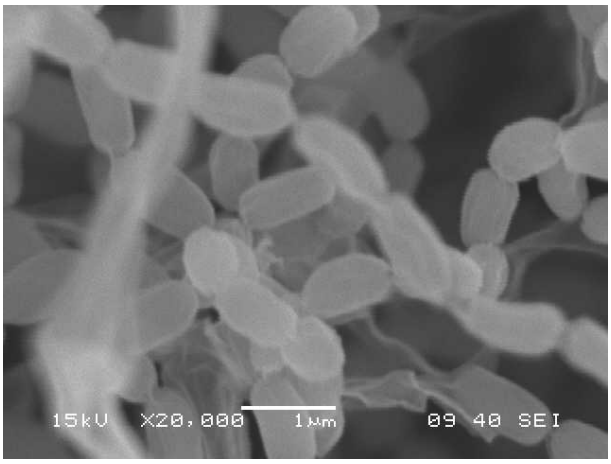
(a)



(b)



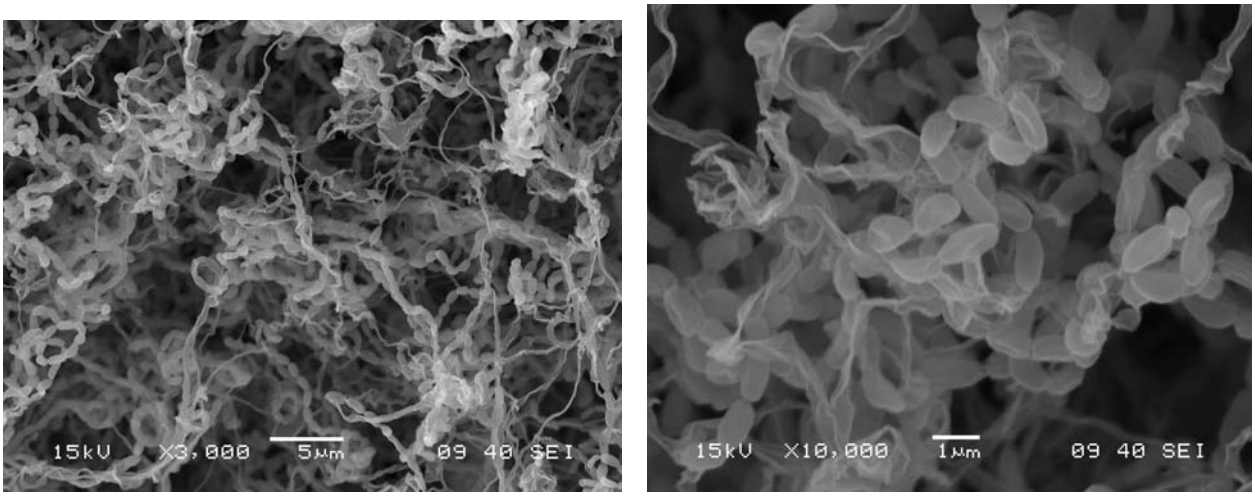
(c)



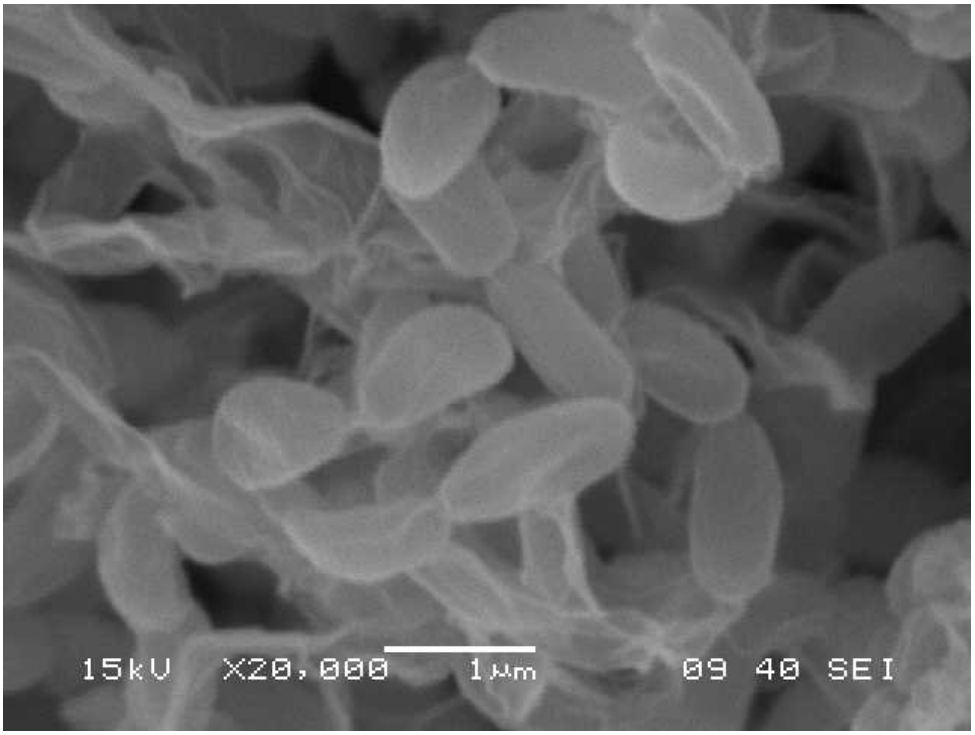
(d)

Figure. 25 (a-d) Results of SEM at different magnification of strain B2.

Strain B2 has shown smooth spore surface morphology. This is the most common form shown by actinomycetes.



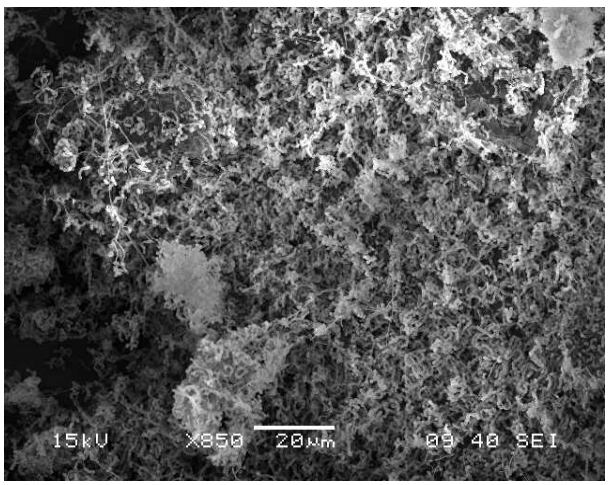
(a) (b)



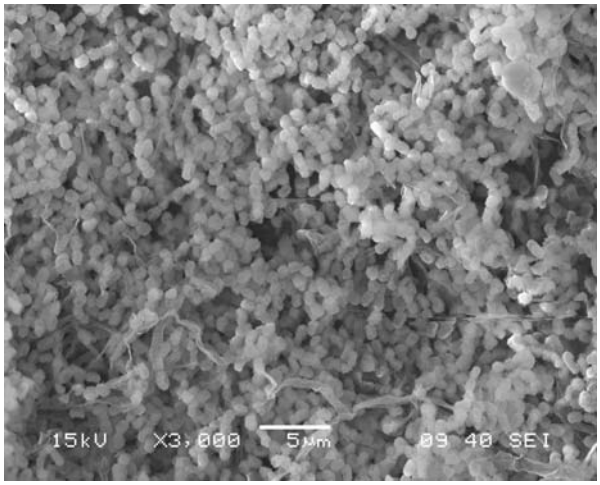
(c)

Figure. 26 (a-c) Results of SEM at different magnification of strain C11.

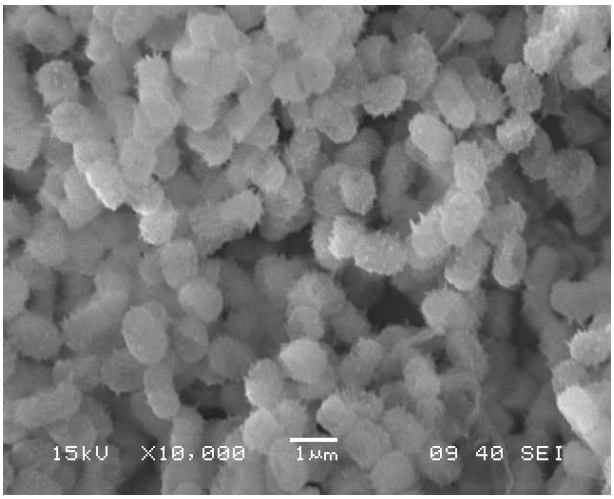
This strain has also shown the smooth spore surface morphology.



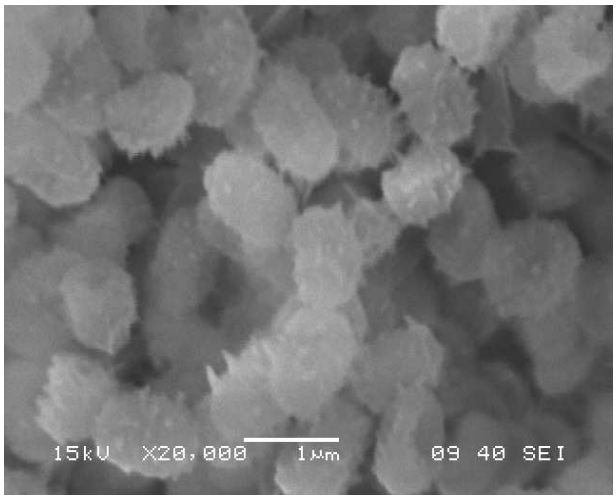
(a)



(b)



(c)



(d)

Figure.27 (a-d) Results of SEM at different magnification of strain CS

This strain has shown different result form the above two strains. Strain was observed as spiny spore surface under different magnification from x850 to x20,000.

f) Screening for antimicrobial activity

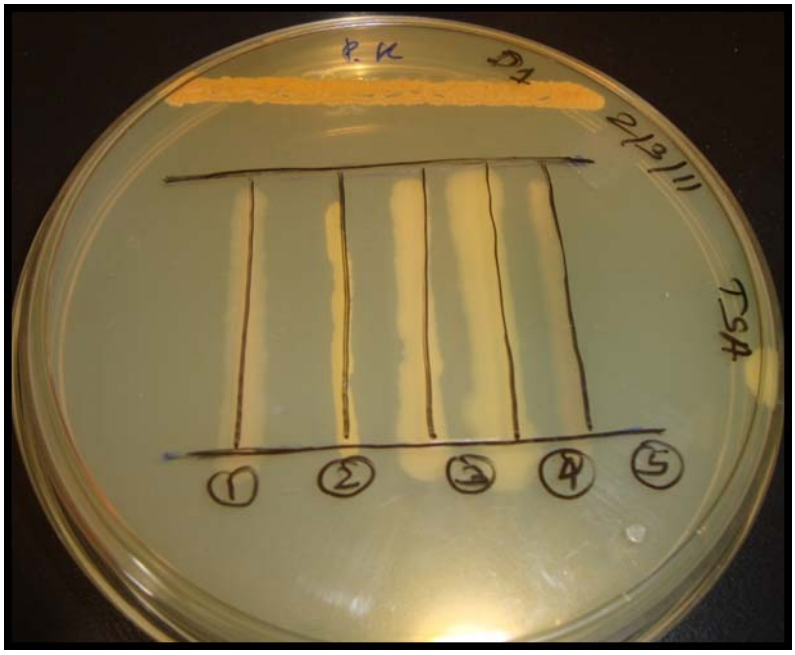
This study was done by the Cross streak method against five pathogenic strains of bacteria namely *Pseudomonas, E.coli, Klebsiella, Bacillus and Proteus* results shown in Table no 9 and Fig:28(a-b).

Table no. 9 General observation of actinomycetes for antimicrobial activity.

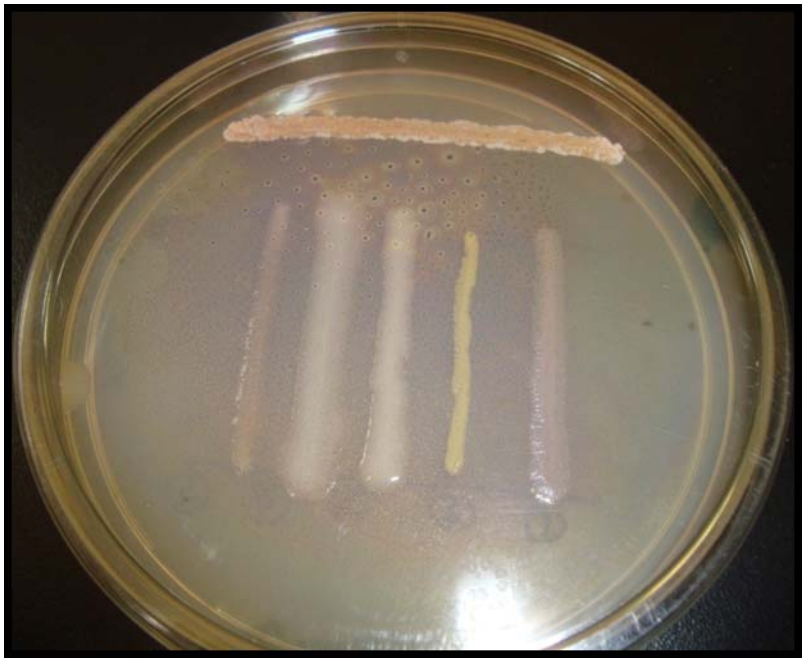
Sl.no	STRAIN	*Pseudomonas*	*E.coli*	*Klebsiella*	*Bacillus*	*Proteus*
1	B2	-	-	-	-	+
2	BS	-	-	+	+	-
3	CO	-	+	-	+	-
4	CS	±	-	-	+	+
5	C1	±	-	-	±	+
6	C8	-	-	+	+	+
7	C9	-	+	+	±	+
8	C11	±	+	+	+	+
9	C12	-	-	+	+	-
10	D1	±	-	++	_	++
11	MB1	-	-	-	-	+
12	MB2	-	-	+	-	+
13	MB3	-	-	+	-	+
14	MB5	-	+	-	-	-
15	MB9	-	+	+	+	+

++: good +: Positive -: Negative ±: Moderate

Among all the strains only D1 has shown good antimicrobial activity against *Klebsiella* and *Proteus.* Growth of *Proteus* was inhibited by most of the strains. MB1 has shown activity against Proteus only. *Pseudomonas was* the least inhibited pathogenic strain CS, C11, C1 and D1 has shown moderate inhibition against it. E.coli has also shown very least resistance against any strains.



(a)



(b)

Figure. 28 (a-b) Screening of antimicrobial activity by cross streak method against five bacterial pathogenic strains.

2. SPECIES AFFILIATION- PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS

a) Assimilation of carbon sources

The ability of different actinomycetes strains in utilizing various carbon compounds as source of energy (Table no 10) was done by following the method recommended in International Streptomyces Project (shown in Fig: 29 a-i).

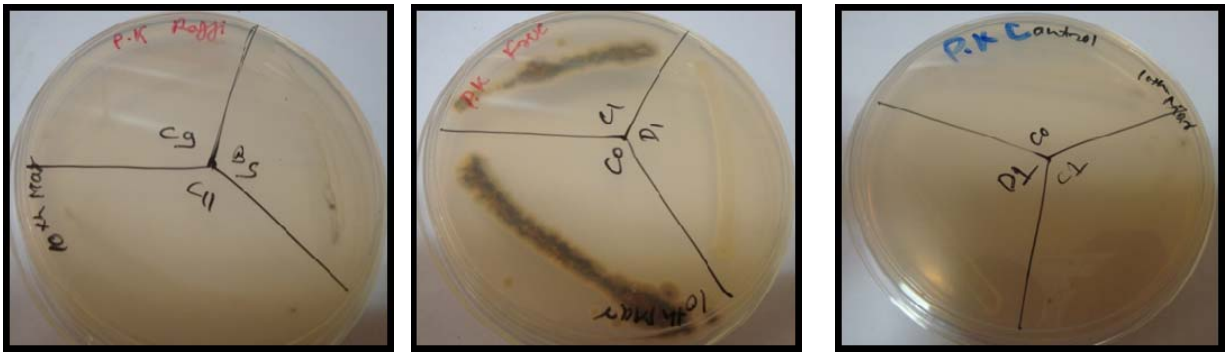
Table no. 10 Results showing assimilation of carbon sources

Sl.no	STRAIN	NEGATIVE CONTROL NO CARBON SOURCE	POSITIVE CONTROL GLUCOSE	XYLOSE	INOSITOL	SUCROSE	RAFFINOSE	FRUCTOSE	RHAMNOSE	MANNITOL	ARABINOSE
1	B2	+	+	+	+	-	-	±	-	+	-
2	BS	+	+	+	+	+	±	+	+	+	±
3	CO	+	+	+	+	+	±	+	+	+	±
4	CS	+	+	+	+	+	+	±	+	+	±
5	C1	+	+	+	+	+	±	+	+	+	±
6	C8	+	+	+	+	+	-	+	+	+	±
7	C9	+	+	+	+	+	+	+	+	+	+
8	C11	+	+	+	+	+	+	+	+	+	±
9	C12	+	+	+	+	+	-	+	+	+	±
10	D1	+	+	+	-	+	±	+	+	+	±
11	MB1	-	-	-	+	-	-	-	++	+	-
12	MB2	-	+	-	+	-	-	-	+	+	±
13	MB3	-	-	-	+	-	-	-	+	+	-
14	MB5	-	-	+	+	-	-	-	+	+	-
15	MB9	+	+	+	+	+	±	+	+	+	±

+++: very good ++: Good +: Positive -: Negative ±: Moderate

(a) (b) (c)

(d) (e) (f)



(g) (h) (i)

Figure.29 (a-i) Assimilation of different carbon sources

After compairing growth with negative and positive control, it was observer that Mannitol was the most assimilated carbon source by all strains of actinomycetes. And the Arabinose was least assimilated carbon source. Among all the strains BS, CO, CS, C1, C9, C11 and MB9 have growth on all carbon sources. MB1, MB2,

MB3 and MB5 have shown least utilization of the carbon sources. But MB1 has shown very good assimilation of Rhamnose as a carbon source in comparison to other strains as well as other carbon sources.

b) Sodium chloride tolerance test

This test was very important for to understand the native nature of the marine actinomycetes isolates. Test result was obtained after 7 to 15 days of incubation. Growth was compared with the control plate. The results obtained were shown in Table no 11.

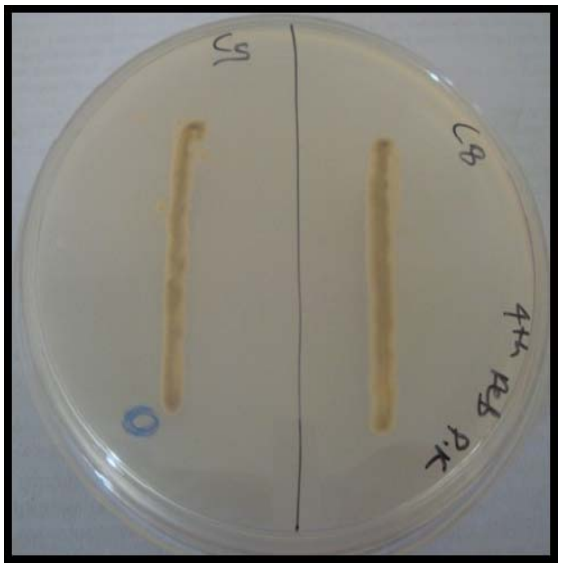
Table no. 11- Results of sodium chloride tolerance test at different concentrations.

Sl.no	STRAIN	5%	10%	15%	20%	25%	30%
1	B2	+	-	-	-	-	-
2	BS	+	±	-	-	-	-
3	CO	+	±	-	-	-	-
4	CS	+	-	-	-	-	-
5	C1	+	-	-	-	-	-
6	C8	+	±	-	-	-	-
7	C9	+	-	-	-	-	-
8	C11	+	-	-	-	-	-
9	C12	+	-	-	-	-	-
10	D1	±	-	-	-	-	-
11	MB1	+	±	-	-	-	-
12	MB2	+	-	-	-	-	-
13	MB3	+	-	-	-	-	-
14	MB5	+	-	-	-	-	-
15	MB9	+	-	-	-	-	-

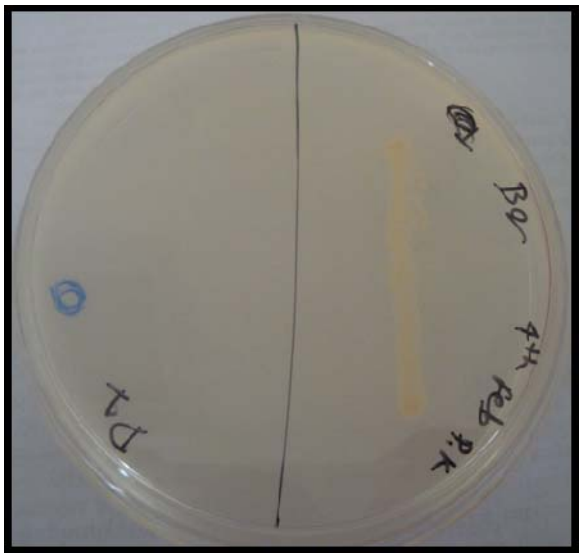
+: Positive -: Negative ±: Moderate

Tolerence was observed only at 5% concentration and some moderate result in 10%. D1 has shown moderate tolerance of sodium chloride. BS, CO, C8 and MB1 strain has shown better tolerance of sodium chloride in comparison to other strains (Fig:30 a-d).

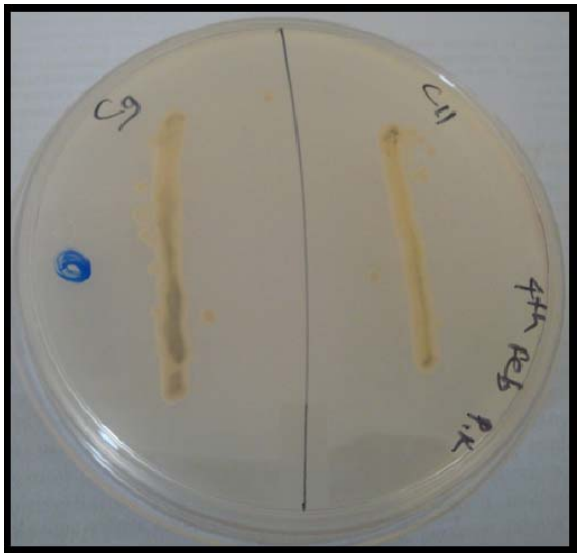
(a)



(b)



(c)



(d)

Figure 30 (a-d) Plates showing the sodium chloride tolerance test

C) Degradation of cellulose

Cellulose degradation was observed for the study the activity of cellulose enzyme shown in Table no 12.

Table no. 12 Results of degradation of cellulose

Sl.no	STRAIN	CELLULASE ACTIVITY
1	B2	++
2	BS	+
3	CO	+
4	CS	+
5	C1	+
6	C8	+
7	C9	+
8	C11	+
9	C12	+
10	D1	+
11	MB1	++
12	MB2	+++
13	MB3	+
14	MB5	+++
15	MB9	++

+++: very good ++: Good +: Positive -: Negative ±: Moderate

Almost all the strains have shown good degradation of cellulose. MB2 and MB5 have shown very good activity of cellulose enzyme than B2, MB1 and MB9. Other strains like BS, CO, CS, C1, C8, C9, C11, C12, D1 and MB3 have shown not very good but positive degradation of cellulose (Fig: 31 a-d).

(a)

(b)

Figure. 31(a-b) Plates showing the cellulose degradation

d)Hydrogen sulphide production

H_2S was observed after the incubation of 7^{th} 10^{th} and 15^{th} day. By compairing the presence of bluish black and black colour slants to the control slants observation were taken (Table no 13).

Table no. 13 Results showing the production of H_2S.

Sl.no	STRAIN	H_2S production
1	B2	+
2	BS	+
3	CO	+
4	CS	+
5	C1	+
6	C8	+
7	C9	-
8	C11	+
9	C12	+
10	D1	+
11	MB1	-
12	MB2	-
13	MB3	-
14	MB5	-
15	MB9	-

+: Positive -: Negative ±: Moderate

Among 15 strains of actinomycetes 9 strains have shown the positive result for the production of H_2S. Six strains namely C9, MB1, MB2, MB3, MB5 and MB9 have not shown positive result for the H_2S production (Fig: no 32 a-b).

(a)

(b)

Figure.32 (a-b) Slants showing the H_2S production by actinomycetes

d) Gelatin liquefication test

This test was done to study the activity of enzyme gelatinase. The slants were observed after the incubation of 7 to 10 days (Fig:no 33). All the result of gelatin liquefication test was negative (Table no. 14) .

Table no14. Results of gelatin liquefication test

Sl.no	STRAIN	GELATINASE ACTIVITY
1	B2	-
2	BS	-
3	CO	-
4	CS	-
5	C1	-
6	C8	-
7	C9	-
8	C11	-
9	C12	-
10	D1	-
11	MB1	-
12	MB2	-
13	MB3	-
14	MB5	-
15	MB9	-

+: Positive -: Negative ±: Moderate

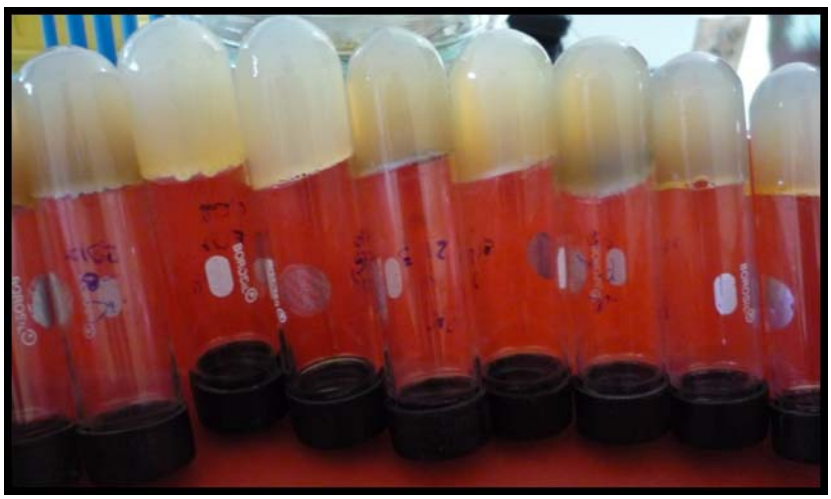


Figure.33 Slants showing the results of gelatin liquefication test

e) Hydrolysis of Starch

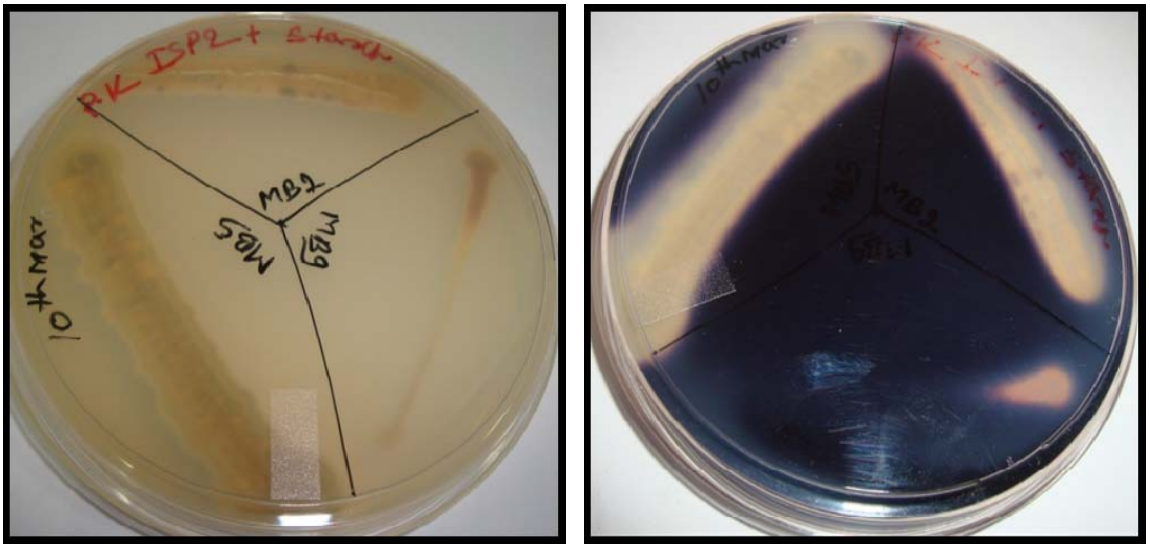
This test was done to observe the activity of amylase enzyme. Almost all strains have shown good amylase activity shown in Table no 15.

Table no 15. Results of Starch hydrolysis by amylase enzyme

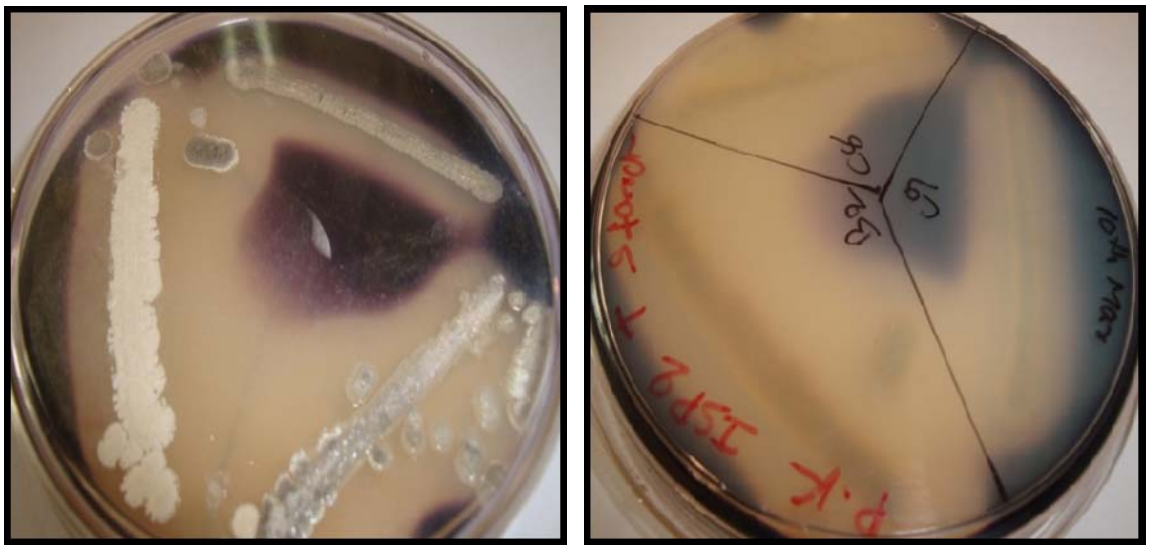
Sl.no	STRAIN	AMYLASE ACTIVITY
1	B2	+++
2	BS	+
3	CO	-
4	CS	+
5	C1	+
6	C8	+++
7	C9	+++
8	C11	±
9	C12	+
10	D1	-
11	MB1	+++
12	MB2	+
13	MB3	+
14	MB5	+++
15	MB9	-

+++: very good ++: Good +: Positive -: Negative ±: Moderate

Satrch hydrolysis was observed by the area of clear zone around the culture streak. Among all strains CO, D1 and MB9 have not shown the amylase activity. Strains namely B2, C8, C9, MB1 and MB5 have shown very good Hydrolysis of Starch. Moderate hydrolysis was shown by the C11. In comparision to that BS, CS, C1, C12, MB2 and MB3 have shown positive activity of amylase enzyme (Fig:no 34).



(a) (b)



(c) (d)

Figure 34 Plates showing the Hydrolysis of Starch

(a) Plate before staining. (b - d) Plates after staining

g) Coagulation of milk

This test was done to study the activity of caseinase enzyme. The slants were observed after the incubation of 7 to 10 days shown in Table no 16.

Table no. 16 Results Showing the coagulation of milk.

Sl.no	STRAIN	CASEINASE ACTIVITY
1	B2	+
2	BS	+
3	CO	-
4	CS	±
5	C1	+
6	C8	++
7	C9	++
8	C11	-
9	C12	++
10	D1	+
11	MB1	-
12	MB2	-
13	MB3	+
14	MB5	±
15	MB9	++

++: Good +: Positive -: Negative ±: Moderate

Among all the strains C12, C8, C9, and MB9 have shown good caseinase activity. At the same time the strains B2, BS, C1, D1 and MB3 were showing positive coagulation of milk. Observation was taken by the comparision of the growth to the control slant. Cs and MB 5 have shown moderate activity of caseinase enzyme. Negative results were shown by the strains Co, C11, MB1 and MB2 (Fig no 35 a-b).

(a)

(b)

Figure. 35 (a - b) Slants showing the coagulation of Milk

h)Ability to grow in different pH .

Almost all the strains have shown pH tolerance at different ranges of pH 5 to pH 9 shown in Table no 17.

Table no. 17 Results showing the growth of actinomycetes on different ranges of pH.

Sl.no	STRAIN	PH 5	PH 6	PH 7	PH 8	PH 9
1	B2	+	+	+	+	+
2	BS	-	+	+	+	+
3	CO	+	+	+	+	+
4	CS	+	+	+	+	+
5	C1	+	+	+	-	-
6	C8	+	+	+	+	+
7	C9	+	+	+	-	-
8	C11	+	+	+	+	-
9	C12	+	+	+	+	+
10	D1	-	-	+	+	-
11	MB1	+	+	+	+	-
12	MB2	+	+	+	-	-
13	MB3	+	+	+	-	-
14	MB5	+	+	+	+	-
15	MB9	+	+	+	-	-

+: Positive -: Negative

Almost all the strains have shown good growth on different ranges of pH. Strains like B2, CO, CS, C8 and C12 have shown growth on each range of pH. This shows there tolerance to grow in acidic as well as basic conditions. At range of pH

5 only strain D1 and BS has shown negative result. At the range of pH 9 B2, BS, CO, CS, C8 and C12 have shown positive results (Fig 36 a-b).

(a)

(b)

Figure.36 (a-b) Slants showing the ph test of marine actinomycetes.

3. Lipolytic activity

Lipolytic activity was studied to observe the enzymatic activity of lipase enzyme (Table no 18).

Table no. 18 Results showing the lipolytic activity of actinomycetes.

Sl.no	STRAIN	LIPOLYTIC ACTIVITY
1	B2	-
2	BS	+
3	CO	-
4	CS	-
5	C1	-
6	C8	-
7	C9	-
8	C11	-
9	C12	-
10	D1	-
11	MB1	++
12	MB2	-
13	MB3	-
14	MB5	-
15	MB9	-

++: Good +: Positive -: Negative ±: Moderat

Among all the strains only MB1 has shown good lipolytic activity and BS has shown some amount of lipase activity.

(a) (b)

Figure.37 (a-b) Plates showing the clear hollow zone of lipolytic activity of strain MB1

After obtaining all the results from the experiment done were matched with the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project) and the species identification was done, the results obtained are given below. The match was done on the basis of maximum percentage of resemblance of characteristics.

Table no.19 Results obtained from the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project)

Sl.no	STRAIN	Species name	ISP description	Page
1	B2	*S.almquisti*	IV	403
2	BS	*S.vastus*	IV	490
3	CO	*S.alni*	V	303
4	CS	*S.luteogriseus*	V	317
5	C1	*A.longiporus*	III	337
6	C8	*A.malachitorectus*	IV	450
7	C9	*S.neyagawaensis*	V	323
8	C11	*A.aureocirculatus*	IV	406
9	C12	*A.janthinus*	III	337
10	D1	*S.spheroides*	III	378
11	MB1	*S.albulus*	V	271
12	MB2	*S.antibioticus*	III	292
13	MB3	*S.mirabilis*	V	321
14	MB5	*S.umbrosus*	IV	486
15	MB9	*S.thermovulgaris*	IV	485

DISCUSSION

Sediment samples collected from the Bhitarkanika mangroves were divided in to two parts as wet sample and dry sample. Serial dilution of wet and dry was done the no of isolates from the dry sample was more in number. Marine sediment samples are good for the isolation of actinomycetes; Goodfellow and Hynes (1984) reviewed the literature on isolation of actinomycetes from marine sediments and suggested that the marine sediment may be valuable for the isolation of novel actinomycetes. 15 isolates of actinomycetes was isolated from the samples. The characterization was done by the phenotypic characterization and species affiliation by physiological and biochemical characteristics described by Das *et al.* (2008).

The aerial mass colour of almost all strains were Whitish grey and only one strain D1 has shown yellow colour. Vanajakumar *et al*. (1995) have also reported that white colour series of actinomycetes they were the dominant forms. Colour series were also recorded in soil, morphological observation of colonial characteristics such as amount and colour of vegetative growth, and the presence and colour of aerial mycelium and spores, and again the presence of diffusible pigments are recorded for each strain studied Colonial growth on agar plate (Labede., 1985).

All strains have shown the pigment formation seven strains have shown the formation of melanoid. The Spore chain morphology was studied by considering different types of spore of actinomycetes under the electron microscope. Spore surface morphology was studied by Scanning electron microscope on three strains among them one strain has shown spiny and of them has shown smooth surface. SEM analysis is very much specific many studies reported spore surface morphology (Sourav and Kannabiran, 2010).

The strains have undergone antimicrobial activity for screening. Mainly strain D1 has shown good inhibition zone against *Klebsiella* and *Proteus.* Almost all strains have shown inhibition against *Proteus* and towards *E. coli* have shown less inhibition. It is observed that the new drugs, notably antibiotics, are urgently needed to halt and

reverse the relentless spread of antibiotic resistant pathogens which use to cause life threatening infections and risk which are undetermining with the viability of healthcare systems (Talbot *et al*., 2006). Filamentous bacteria belonging to the order *Actinomycetales*, especially *Micromomospora* and *Streptomyces* strains, have a unique and proven capacity to produce novel antibiotics (Bentley *et al.,* 2002), hence the continued interest in screening such organisms for new bioactive and It is also becoming increasingly clear that un- and under-explored habitats, such as desert biomes and marine ecosystems, are a very rich source of novel actinomycetes which have the capacity to produce interesting new bioactive compounds, including antibiotics (Hong *et al*., 2009).

Another study done by Jeffrey *et al.* (2007) shown the antagonistic activity of actinomycetes against three strains of pathogenic microbes (*Fusarium palmivora, Bacillus subtilis, Ralstonia solanacearum* and *Pantoae dispersa*). All the strains were chosen due to the reason that these microbes exhibited pathogenic effect towards certain commodity plants. Antimicrobial tests showed that 3, 25, 37 and 35 isolates of actinomycetes produces antagonistic reaction for *F. palmivora, B. sub.*

By all these discussion it can be said as those actinomycetes produces some useful bioactive compounds. All this screening is done by the coverslip culture technique as also done by Thenmozhi and Kannabiran (2010). The spore chain and spore surface morphology were analyzed by direct microscopic examination of the culture area by cover slip culture technique. Utilization of carbon sources arabinose, xylose, inositol, mannitol, fructose, rhamnose, sucrose and raffinose were analysed for classification. These carbon sources were separately supplemented (1%) in each ISP 1 medium.

Another major milestone in the identification of actinomycetes was the assimilation of carbon by actinomycetes. Test includes ten carbon sources which are sterilized by membrane filtration method. Almost all the strains have shown very luxuriant growth. Some of the strains MB1, MB2, MB3, and MB5 have shown less growth. Pandey *et al.,* (2005) showed that for the optimum production of antibiotics certain carbon sources are required. In that study the author and coworkers also suggested that pH might play an important factor for the production of antibiotics by

actinomycetes. pH test done on all the 15 atrains has shown positive results. As the sample are collected from the mangroves, so it is quite expected that strains can tolerate high diversification in salinity. Study done by Vasavada et al. (2006) showed that the used of media, pH, salinity and carbon and nitrogen affect the growth and antibiotic production by actionmycetes. Some of the studies reported that the morphology of the spore bearing hyphae with entire spore chain along with substrate and aerial mycelium was examined under light microscope as well as scanning electron microscope. Carbohydrate utilization was determined by growth on carbon utilization medium (ISP 9) (Pridham and Gottlieb, 1948) supplemented with 1% carbon source at 28 °C. Temperature range for growth was determined on inorganic salt starch agar medium (ISP 4) by growing at different temperatures (for example, 15°, 25°, 37°, 42° and 50 °C). Hydrolysis of starch was evaluated by using the media of Gordon *et al*., (1974) Liquefaction of gelatin was evaluated by the method of Waksman (1961). H_2S production test has been done by preparing the slant culture among all the strains nine strain have shown positive result.

To know the overall activity of all the strains various enzymatic screening has been done. These are the cellulose activity, caseinase activity, amylase activity, lipolytic activity and gelatinase activity. Gelatin hydrolysis was not shown by any strain. But the amylase activity is shown by almost all the strains. Cellulose and caseinase activity is shown almost in the same manner by all strains. Al these results has shown same pattern of results obtained by previous works done by many researchers.

Finally, after all these experiments results have been matched with the keys given for 458 species of actinomycetes included in ISP (International Streptomyces Project) and the species identification was done and it was found that all the isolates have been grouped under *Streptomyces* genus.

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